Investigating the Isomer-specific Effects of Conjugated Linoleic Acid on Metabolic Syndrome, Hepatic Steatosis and Selected Mediators of Hepatic Lipid Metabolism in the *fa/fa* Zucker Rat

By

Danielle Marie Stringer

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

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

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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

Investigating the Isomer-specific Effects of Conjugated Linoleic Acid on Metabolic Syndrome, Hepatic Steatosis, and Selected Mediators of Hepatic Lipid Metabolism in the *fa/fa* Zucker Rat

Danielle M. Stringer, M.Sc. Thesis,

Department of Human Nutritional Sciences

Metabolic syndrome (MetS) is a condition characterized by insulin resistance, glucose intolerance, hyperlipidemia, obesity, and hypertension. Past research has shown that conjugated linoleic acid (CLA) can improve the conditions associated with MetS, including hepatic steatosis, a newly-emerging pathology of MetS. Isomer-specific studies indicate that *trans*-10, *cis*-12 (t10,c12) CLA improves insulin sensitivity and glucose tolerance in the *fa/fa* Zucker rat, while *cis*-9, *trans*-11 (c9,t11) CLA has neutral effects. Therefore, we hypothesized that t10,c12 CLA would improve hepatic steatosis, liver function, and inflammation associated with MetS.

To test this hypothesis, 6-week-old male *fa/fa* and lean Zucker rats were randomly assigned to consume one of five treatment diets containing c9,t11 CLA alone, t10,c12 CLA alone, or c9,t11 and t10,c12 CLA in combination. All diets contained 0.4% (wt/wt) of c9,t11 and/or t10,c12 CLA. After 8 weeks of feeding, dietary t10,c12 CLA reduced hepatic steatosis by 61% in *fa/fa* rats. This

reduction was not associated with higher activation of AMP-activated protein kinase or reduced activation of sterol regulatory element-binding protein-1, two key mediators of lipid metabolism in the liver. However, reduced hepatic steatosis was associated with improved liver function, as evidenced by 48% reduction in serum alanine aminotransferase. Serum haptoglobin, a marker of inflammation, was reduced by 27% when *fa/fa* rats were fed t10,c12 CLA.

Results from this study suggest that t10,c12 CLA could be useful in treating and managing MetS. However, the efficacy of CLA for this purpose in the human population requires further investigation.

ACKNOWLEDGEMENTS

I would like to sincerely thank my two advisors, Dr. Carla Taylor and Dr. Peter Zahradka, for their guidance, patience, encouragement and support. They have been excellent mentors and teachers and I thank them for allowing me to be a part of their labs. Thank-you also to Dr. Miyoung Suh and Dr. Thomas Netticadan for being a part of my committee. Their input is greatly appreciated.

Thank-you to Laura Burr, Vanessa DeClercq, Rob Diakiw, Dielle Herchak, Jennifer Jamieson, Ling Lee, Lisa Maximiuk, Amy Noto, Lisa Rigaux, Natasha Ryz, and Jennifer Zahradka for assistance with animal work and laboratory analyses. Thank-you also to Brenda Wright, Xueping Xie and Melissa Fuerst for assistance and advice with molecular work.

The statistical analyses for this project were at times quite a challenge, and could not have been completed without the help of Dan Chateau and Dr. Ken Mount. Their expertise and knowledge are greatly appreciated.

I would also like to thank all my fellow graduate students for being a great support network. Thanks also to the staff and faculty of the Department of Human Nutritional Sciences and Faculty of Human Ecology.

Lastly, thank-you to all my friends and family for being so understanding over the past two years. A special thank-you goes to my parents, Rene and Genevieve Defries, and my husband Michael Stringer, for all their love and faith in me.

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ABBREVIATIONS

AACE	American Association of Clinical Endocrinology
ACC	acetyl-CoA carboxylase
ACO	acyl-CoA oxidase
AICAR	5-aminoimidazole-4-carboxamide ribofuranoside
ALT	alanine aminotransferase
AMPK	AMP-activated protein kinase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
BCA	bis-cinchinonic acid
BIO	Bioriginals diet
BMI	body mass index
BSA	bovine serum albumin
c9,t11	*cis*-9, *trans*-11
ChREBP	carbohydrate response element binding protein
CLA	conjugated linoleic acid
CPT-1	carnitine palmitoyl transferase-1
CTL	control diet
ddH_2O	distilled, deionized water
EDTA	ethylene diamine tetraacetic acid
EGSIR	European Group for the Study of Insulin Resistance
ER	endoplasmic reticulum
FAS	fatty acid synthase

FFA	free fatty acids
GK	glucokinase
GKRP	glucokinase regulatory protein
glc 6-Pase	glucose 6-phosphatase
GSK3α/β	glycogen synthase kinase-3α/β
H_2O_2	hydrogen peroxide
HDL	high-density lipoprotein
HMG-CoA synthase	hydroxymethylglutaryl-CoA synthase
IL-6	interleukin-6
IRS	insulin receptor substrate
^{125}I	^{125}Iodine
JNK	c-Jun NH_2-terminal kinase
MAPK	mitogen activated protein kinase
MB	maximum binding
MetS	metabolic syndrome
NCEP	National Cholesterol Education Program
OGTT	oral glucose tolerance testing
$pAkt^{ser}$	phosphorylated Akt on serine residues
$pAkt^{thr}$	phosphorylated Akt on threonine residues
pAMPKα	phosphorylated AMPKα
pGSK3α/β	phosphorylated GSK3α/β
$pIRS\text{-}1^{tyr}$	phosphorylated IRS-1 on tyrosine residues
pMAPK	phosphorylated MAPK

NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NCK	NuCheck Prep diet
NSB	non-specific binding
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PEPCK	phosphoenolpyruvate carboxykinase
PI3K	phosphatidylinositol 3'-kinase
PKB	protein kinase B
PPAR	peroxisome proliferator activated receptor
PPRE	peroxisome proliferator response element
PtdIns $(4,5)P_2$	phosphotidylinositol (4,5) bisphosphate
PtdIns $(3,4,5)P_3$	phosphotidylinositol (3,4,5) trisphosphate
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene
PW	pair-weighed group
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SH_2	SRC homology
SOCS	suppressor of cytokine signaling
SREBP	sterol regulator element-binding protein
SREBP-1 p68	activated SREBP-1
SREBP-1 p125	full-length SREBP-1
TAG	triacylglycerol

TBHQ	tert-butyl hydroquinone
TBST	tris-buffered saline with Tween-20
TC	total counts
TEMED	N, N, N', N'-Tetramethylethylenediamine
TNF-α	tumor necrosis factor-α
TOG	Together diet
t10,c12	*trans*-10, *cis*-12
t=0	timepoint 0 minutes
t=15	timepoint 15 minutes
t=30	timepoint 30 minutes
t=60	timepoint 60 minutes
RIA	radioimmunoassay
RXR	retinoic acid receptor
VLDL	very-low density lipoprotein
WHO	World Health Organization
wt/wt	weight by weight
wt/v	weight by volume
ZDF	Zucker diabetic fatty
9-11	c9,t11 diet
10-12	t10,c12 diet

LITERATURE REVIEW

Introduction

Conjugated linoleic acid (CLA) is a term that includes all geometric and positional isomers of the eighteen-carbon fatty acid linoleic acid (Pariza et al., 2001; Banni, 2001). Among the numerous potential heath benefits of CLA is its ability to improve aspects of the metabolic syndrome (MetS), a condition characterized by the presence of impaired glucose tolerance, insulin resistance, hyperlipidemia, hypertension, and obesity (Aminot-Gilchrist & Anderson, 2004). As obesity increases in North American adults and children there is a parallel increase in the prevalence of MetS among both these age groups, which is imposing an increasingly heavy financial burden on the health care system (Pierce, 2003). In addition to its favourable effect on classic characteristics of MetS, CLA reduces hepatic steatosis, a condition that is now considered the hepatic consequence of MetS (Nagao et al., 2005; Noto et al., 2006; Marchesini et al., 2001). The purpose of this literature review is to describe the potential role for CLA as a nutraceutical agent for management of MetS, with a specific focus on hepatic steatosis and selected molecular regulators of lipid metabolism that may be involved in the anti-steatotic effects of CLA.

Metabolic Syndrome

Although MetS was first described in 1988 by Reaven (Reaven, 1988) the associations between the characteristics of MetS have been noted since as early as the beginning of the century (Sarafidis & Nilsson, 2006). Since that time, many names have been given to the cluster of metabolic abnormalities inherent of MetS, including metabolic trisyndrome, syndrome X, deadly quartet and insulin resistance syndrome.

MetS, as it is widely called today, is characterized by the coexistence of certain pathologies, including abdominal obesity, dyslipidemia, hypertension, insulin resistance, and glucose intolerance. Recent studies have also suggested inflammation as an additional component of MetS (Ridker et al., 2004; Gonzales et al., 2006; Haffner, 2006). Although the main pathologies of MetS are generally recognized by different health-regulatory bodies, the diagnostic criteria are variable. Several health organizations, including the World Health Organization, the National Cholesterol Education Program, the European Group for the Study of Insulin Resistance, and the American Association of Clinical Endocrinology have proposed their own diagnostic criteria for MetS (Table 1) (Alberti & Zimmet, 1998; Cleeman, 2001; Balkau & Charles, 1999; American College of Endocrinology, 2003).

Varying diagnostic criteria make it difficult to estimate the true prevalence of MetS. Using the definition of the National Cholesterol Education Program and

Table 1 – Diagnostic Criteria for MetS

	Blood Pressure	***Plasma Lipids***[5]	***Hyperglycemia***	***Adiposity***	***Insulin Resistance***[6]
WHO[1]	>140/90	TAG ≥ 1.7 mmol/L **or** HDL-C ≤ 0.9 – 1.0 mmol/L	Impaired glucose tolerance or Type 2 diabetes	Body mass index >30 kg/m^2 **or** waist:hip ratio >0.85 (women) – 0.9 (men)	Fasting insulin > 75th percentile **or** HOMA-IR > 75th percentile **or** M value <25th percentile
NCEP[2]	>130/85	TAG ≥ 1.7 mmol/L **and** HDL-C <1.0-1.3 mmol/L	Fasting glycemia >5.5 mmol/L	Waist circumference >102 cm in men and >88 cm in women	Not included
EGSIR[3]	>140/90 or antihypertensive therapy	TAG ≥ 2.0 mmol/L **or** HDL-C <1.0 mmol/L **or** lipid lowering treatment	Fasting glycemia >6.0 mmol/L	Waist circumference >94 cm in men and >80 cm in women	Fasting insulin >75th percentile
AACE[4]	>130/85	TAG ≥ 1.7 mmol/L **and** HDL-C <1.0 – 1.3 mmol/L	Fasting glycemia 6.0-6.9 mmol/L **or** impaired glucose tolerance	Not included	Not included

[1] World Health Organization; diagnosis requires presence of two or more components plus the existence of either Type 2 diabetes, impaired glucose tolerance, or insulin resistance.
[2] National Cholesterol Education Program; diagnosis requires presence of three components.
[3] European Group for the Study of Insulin Resistance; diagnosis requires presence of two components.
[4] American Association of Clinical Endocrinology; diagnosis requires presence of two components.
[5] TAG = Triacylglycerol; HDL-C = High density lipoprotein cholesterol.
[6] HOMA-IR = Homeostasis model assessment index of insulin resistance.

the results of the Third National Health and Nutrition Examination Survey, it was estimated that 47 million people in the United States had characteristics of MetS (Ford, 2004). In Canada, estimates reach as high as 25.8% of the population, or roughly 8 million people; however, prevalence rates among ethnic subsets of the population range from 11% in the Inuit to as high as 45% in First Nations people (Lui et al., 2005).

MetS can occur early in life. Using the diagnostic criteria of the World Health Organization, modified for children and adolescents, Invitti et al. (2006) found that 23.3% of overweight Italian Caucasian children between the ages of 6 and 16 years had MetS. In North America, prevalence of MetS among children and adolescents is higher; however, this difference could be due to evaluation based on a different set of diagnostic criteria. Using data from the Third National Health and Nutrition Examination Survey (1988-1994), Cook et al. (2003) found that 28.7% of overweight adolescents (age 12-19) had MetS. The overall prevalence for this age group was 4.2% (6.1% for males and 2.1% for females). This study also found a relationship between prevalence and degree of obesity: the prevalence in youth with a body mass index (BMI) greater than the 95th percentile was 28.7%, decreased to 6.8% in youth with a BMI between the 85th and 95th percentile, and further decreased to 0.1% in adolescents with a BMI less than the 85th percentile.

Alarmingly, the prevalence of MetS among adolescents is increasing. Based on data collected from the Fourth National Health and Nutrition Examination Survey (1999-2000), the overall prevalence of MetS was 6.4%, or

approximately 2 million adolescents, representing an increase of 2.2% from the period of 1988-1994. MetS was more prevalent in adolescent males (9.1%) than in adolescent females (3.7%). Amongst overweight adolescents, the prevalence of MetS rose to 32.1% from 28.7% in 1988-1994 (Duncan et al., 2004).

Overweight and Obesity

According to the World Health Organization and Health Canada, a person having a BMI of greater than 25 kg/m^2 is considered overweight, while a person having a BMI of greater than 30 kg/m^2 is considered obese (World Health Organization, 1999; Office of Nutrition Policy and Promotion, 2003). The obese classification is further divided into three classes: Class I (BMI 30.0 to 34.9), Class II (BMI 35.0 to 39.9) and Class III (BMI 40.0 or greater). Overweight and obesity are risk factors for a number of diseases, including MetS and Type 2 diabetes (Mokdad et al., 2003). Obese individuals without MetS or Type 2 diabetes can also be insulin resistant.

The rising prevalence of MetS is likely attributable to increasing obesity rates. Regardless of definition or diagnostic criteria, older age, level of physical fitness, and increased percentage body fat are associated with increased risk of MetS (Lui et al., 2006). In 1979, 13.8% of Canadian adults were obese. Results from the recent Canadian Community Health Survey reveal that, presently, 36.1% of adult Canadians have a BMI between 25 and 30, while 23.1% have a BMI greater than 30 (Tjepkema, 2005). Considering rates of overweight and

obesity together, the majority of Canadian adults (almost 60%) are at increased risk for several serious chronic diseases, including Type 2 diabetes, MetS, hypertension, hypercholesterolemia, and cancer (Mokdad et al., 2003). Globally, approximately 1 billion people are overweight, and 300 million people are obese (World Health Organization, 1999).

Pharmacological treatments for obesity are available, and include both peripheral and central-acting medications. Peripheral-acting medications, such as Orlistat, promote malabsorption in the gastrointestinal tract. More numerous are the central-acting anti-obesity medications, derived from β-phenethylamine and phenylpropanolamine, which have anorectic effects. Examples of central-acting anorectic medications include phentetrazine, benzophetamine, fendimetrazin, diethylproprion, mazindol, femproporex, phentermine, fenfluramine, dexfenfluramine, sibutramine, phenylpropanolamin and ephedrine. (Halpern & Mancini, 2003) However, despite the numerous anti-obesity medications available, rates of overweight and obesity are still increasing. Therefore, other diet or lifestyle-based strategies for weight loss should be investigated.

Insulin Resistance

Insulin resistance is defined as a condition whereby the cells of the body become resistant to the effects of the hormone insulin (Groff & Gropper, 2000). Insulin resistance can occur in skeletal muscle, adipose tissue, and the liver.

This insulin resistance may stem from certain risk factors including older age, obesity, excessive caloric intake, and physical inactivity. Although increased age is indicated as a risk factor, recent statistics show increased prevalence of insulin resistance among children and youth, which may be attributed to decreased physical activity and increased obesity rates among this age group (Gabbay et al., 2003; Fagot-Campagna et al., 2000). Along with environmental causes, genetic predisposition is also implicated. The prevalence of Type 2 diabetes, a disease characterized by insulin resistance, is higher in certain minority groups, for example, the Pima Indians of Arizona, and more locally, the First Nations people of Manitoba (Fagot-Campagna et al., 2000).

Insulin Signaling

The insulin receptor is a tetrameric protein belonging to a subgroup of receptor tyrosine kinases (Saltiel & Kahn, 2001). Insulin exerts its numerous hormonal effects by triggering a cascade of phosphorylation reactions inside the target cell. The binding of insulin to its cell surface receptor stimulates intrinsic tyrosine kinase activity of the insulin receptor, causing phosphorylation of tyrosine residues on the receptor and creation of docking sites for intracellular insulin receptor substrates, including a family of 6 insulin receptor substrate (IRS) proteins (Pirola et al., 2004; Saltiel & Kahn, 2001). Although homologous and implicated in normal insulin signaling, IRS-1 is important for the effects of insulin in skeletal muscle, while IRS-2 primarily mediates insulin action in the liver (Previs et al., 2000; Kido et al., 2000). Hepatic IRS-2 expression is decreased in

insulin-resistant mice, and this results in impaired downstream insulin signaling (Kerouz et al., 1997; Shimomura et al., 2000).

Tyrosine phosphorylation of IRS proteins in hepatocytes promotes binding to Src homology (SH_2) domain-containing proteins, including phosphotidylinositol 3'-kinase (PI3K) (Rother et al., 1998; Valverde et al., 2003). PI3K is considered an essential transducer in normal insulin signalling, as negative effects on its expression or activity inhibit most of the cellular responses to insulin (including stimulation of glucose transport as well as glycogen and protein synthesis (Lizcano & Alessi, 2002). Movement of PI3K to the plasma membrane allows close contact with its substrate, phosphotidylinositol (4,5) bisphosphate [PtdIns $(4,5)P_2$], which is then phosphorylated to produce phosphotidylinositol (3,4,5) trisphosphate [PtdIns $(3,4,5)P_3$] (Pirola et al., 2004).

Protein kinase B (PKB, also known as Akt) is an important signalling molecule activated by PtdIns $(3,4,5)P_3$. PtdIns $(3,4,5)P_3$ binds PKB, recruits it from the cytosol and brings it closer to protein kinase 3-phosphoinositide-dependent protein kinase-1/2, which activates PKB by phosphorylation (Lizcano & Alessi, 2002). Upon activation, PKB can translocate throughout the cell, thus activating numerous pathways that play important roles in regulating insulin dependent processes in insulin-responsive tissues, such as glucose transporter translocation to the cell membrane for glucose uptake and glycogen synthesis (White, 2002).

The PI3K pathway also controls metabolism by regulating gene transcription. In the liver, PKB influences forkhead transcription factors which

play a key role in the regulation of gluconeogenic enzymes. For example, the forkhead response element occurs in the genes encoding for two rate-limiting enzymes of gluconeogenesis: phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (glc 6-Pase) (Pirola et al., 2004). When phosphorylated by PKB, activity of forkhead transcription factors is inhibited, and transcription of gluconeogenic enzymes ceases. Thus, binding of insulin to its receptor inhibits gluconeogenesis through PKB-mediated inactivation of forkhead transcription factors

Interruption at any point of the insulin-stimulated intracellular phosphorylation cascade inhibits the numerous metabolic effects of insulin. Although there are many factors influencing insulin signaling, this literature review will focus on the role of altered production of adipocytokines in obesity and how adipocytokines may negatively affect insulin signaling.

Adipocytokines and Insulin Resistance

Adipocytokines are proteins secreted by white adipose tissue. Their synthesis and secretion are altered during changes in white adipose tissue mass, as in obesity (Guerre-Millo, 2003). Tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and resistin, are examples of pro-inflammatory adipocytokines whose production is increased in obesity, whereas adiponectin is an anti-inflammatory adipocytokine whose production is decreased in obesity.

TNF-α

TNF-α is a 26-kDa transmembrane protein, which is released into the circulation as a 17-kDa soluble protein after extracellular cleavage by a metalloproteinase (Gearing et al., 1994). Strong associations exist between TNF-α and insulin resistance. Animal models of obesity with insulin resistance and/or Type 2 diabetes display increased adipose tissue TNF-α expression and serum TNF-α concentration (Hotamisligil et al., 1993; Hofmann et al., 1994; Iwai et al., 2003). Insulin resistance is corrected in these animals upon treatment with soluble TNF-α receptors (Hotamisligil et al., 1993). Increased TNF-α mRNA levels are also seen in livers of insulin resistant animals (Tilg & Diehl, 2000). In peripheral and hepatic tissues, TNF-α can inhibit glucose uptake both in vivo and in vitro (Del Aguila et al., 1999; Hotamisligil et. al, 1994; Spiegelman & Hotamisligil, 1993; Lang et al., 1992). In the *fa/fa* Zucker rat, neutralization of TNF-α leads to a significant improvement in hepatic insulin sensitivity, measured by the complete suppression of hepatic glucose output (Cheung et al., 1998). In humans, elevated serum concentrations of TNF-α have been observed in obese type 2 diabetic patients (Katsuki et al., 1998).

The association between elevated adipose TNF-α mRNA and serum TNF-α concentrations and insulin resistance suggests an inhibitory effect of TNF-α on insulin signaling at the molecular level. Indeed, studies have revealed that TNF-α induces phosphorylation of serine^{307}on IRS-1, inhibiting IRS-1 tyrosine phosphorylation and impairing the insulin signaling cascade at the level of the insulin receptor, with consequent reductions in IRS-1-associated PI3K and PKB

activities (Aguirre et al., 2000; Rui et al., 2001; de Alvaro et al., 2004). Whether TNF-α affects tyrosine phosphorylation of hepatic IRS-2, the major IRS protein in hepatocytes, remains to be determined.

IL-6

IL-6 ranges in size from 22 to 27 kDa, depending on the degree of glycosylation of the molecule (Gross et al., 1989). Studies in humans have shown an association between plasma IL-6 concentrations and insulin resistance and glucose intolerance (Bluher et al., 2005). In healthy participants, plasma IL-6 concentrations are inversely associated with insulin sensitivity (Heliovaara et al., 2005). Studies in hepatocytes show that IL-6 impairs insulin signaling by down-regulation of IRS-1 and upregulation of suppressor of cytokine signaling-3 (SOCS-3), a negative regulator of insulin signaling (Senn et al., 2003). The effects of IL-6 on SOCS-3 expression have also been observed in human adipose tissue and skeletal muscle (Rieusset et al., 2004).

Resistin

Resistin is a 12.5 kDa protein that is synthesized and secreted by adipose tissue. It previously has been assumed that increased serum resistin concentrations in obesity are due to overproduction by adipose tissue itself; however, this increase also may result from production by macrophages that infiltrate adipose tissue during obesity (Weisberg et al., 2003; McTernan et al., 2006). In humans, plasma resistin concentrations correlate with insulin

resistance (Degawa et al., 2003; Azuma et al., 2003). In Sprague Dawley rats, infusion of resistin during a pancreatic insulin clamp increased the amount of insulin required to maintain glucose at basal levels (Rajala et al., 2003). Furthermore, despite the increased infusion of insulin, hepatic glucose production was higher in rats treated with resistin, suggesting impaired hepatic insulin sensitivity. In high-fat fed mice, plasma resistin increased by 80% after 3 weeks, and was associated with a two-fold increase in the rate of endogenous glucose production (Muse et al., 2004). However, when these mice were treated for 1 week with a resistin sequence-specific antisense oligodeoxynucleotide, hepatic insulin resistance was completely reversed. Molecular effects of resistin on insulin signaling may include impairement of insulin receptor phosphorylation, IRS-1 phosphorylation, PI3K activation, and/or PKB activation, possibly by enhancing gene expression of SOCS-3 (Steppan et al., 2005).

Adiponectin

Adiponectin is a 30 kD adipocytokine secreted by white adipose tissue. Adiponectin (also known as Acrp30) found in the serum can be in either the full-length form or a smaller globular form (gAcrp30), which results from proteolytic cleavage of the full-length form (Kadowaki & Yamauchi, 2005). Contrary to the other adipocytokines, serum levels of adiponectin are decreased in obesity and it has anti-inflammatory effects (Arita et al., 1999; Gable et al., 2006). Recently, a study using adiponectin knockout mice found that TNF-α and adiponectin each negatively affect expression of the other. The knockout mice had increased

levels of TNF-α in adipose tissue and increased plasma TNF-α concentrations, both of which were reduced following infusion of adiponectin (Xu et al., 2003).

Serum concentrations and adipose levels of adiponectin mRNA are significantly decreased in animal models of insulin resistance (Hotta et al., 2000; Yamauchi et al., 2001). In particular, decreased plasma adiponectin concentrations correlate with increased insulin resistance in *fa/fa* Zucker rats (Altomonte et al., 2003). Longitudinal studies using rhesus monkeys provide evidence of a strong correlation between circulating adiponectin levels and insulin sensitivity. In this animal model, plasma adiponectin concentrations decreased at the early stages of obesity and continually decreased after the development of Type 2 diabetes (Hotta et al., 2001). In human populations, plasma concentrations of adiponectin are inversely associated with BMI, fasting insulin levels, and insulin resistance (Weyer et al., 2001; Shand et al., 2003). Administration of recombinant adiponectin lowers serum glucose and improves insulin resistance in murine models of obesity and diabetes (Tsao et al., 2002; Berg et al., 2002). Mice deficient in adiponectin display moderate to severe insulin resistance with reduced IRS-1–associated PI3K activity and glucose uptake (in muscle); administration of adiponectin ameliorates these problems (Kubota et al., 2002; Maeda et al., 2002). These results indicate a possible metabolic function of adiponectin in insulin resistance; however, it is unknown whether these improvements in insulin sensitivity result from improved insulin signaling in adipose, skeletal muscle, or liver tissue, or a combination of all three tissues.

Using *ob/ob* C57BL mice, Berg et al. (2001) found that acute doses of recombinant adiponectin ameliorated hepatic insulin resistance, as shown by suppression of hepatic glucose output. Isolated rat hepatocytes treated with adiponectin also displayed decreased glucose output (Berg et al., 2001). Purified recombinant adiponectin infused into mice during a pancreatic euglycemic clamp has also been shown to decrease hyperglycemia by suppression of hepatic glucose output, and also to decrease hepatic expression of PEPCK and glc 6-Pase (Combs et al., 2001). These results support the hypothesis that adiponectin improves insulin sensitivity, in part, by improving hepatic insulin signaling.

Overall, simultaneous increases in circulating proinflammatory adipocytokines and decreases in circulating anti-inflammatory adipocytokines observed in obesity may play a role in the development of insulin resistance. Therefore, pharmacological or dietary interventions aimed at favourably modifying adipocytokine production could be helpful in the treatment of insulin resistant states such as MetS.

Effects of Insulin Resistance on the Liver

Accumulation of lipid in the liver is the hepatic consequence of insulin resistance (Stone & Van Thiel, 1985; Van Steenbergen & Lanckmans, 1995). Up to 75% of patients with Type 2 diabetes have some degree of fatty liver (Neuscwander-Tetri & Caldwell, 2003). Insulin resistance in adipose tissue leads to unregulated activity of hormone sensitive lipase, which increases adipose

lipolysis and release of free fatty acids (FFA) into the bloodstream. In response, hepatic FFA uptake increases, leading to augmented hepatic esterification of FFA to triacylglycerol (TAG) and enhanced oxidation to ketone bodies. When the rate of hepatic fatty acid uptake exceeds the capacity for oxidation or secretion of very-low density lipoprotein (VLDL), the liver stores any excess TAG (Gibbons, 1990; Van Steenbergen & Lanckmans, 1995).

Levels of certain hormones also influence hepatic fatty acid metabolism. Insulin inhibits mitochondrial β-oxidation of fatty acids, while stimulating synthesis of fatty acids from glucose and synthesis of TAG from fatty acids (Van Steenbergen & Lanckmans, 1995; Gibbons, 1990). In addition, elevated levels of glucagon may act to decrease VLDL secretion, furthering hepatic steatosis (Stone & Van Thiel, 1985). Although previously considered benign, hepatic steatosis does have an impact on the degree of hepatic insulin resistance, leading to increased gluconeogenesis and decreased glycogen synthesis that contributes to hyperglycemia (Samuel et al., 2004).

Non-Alcoholic Fatty Liver Disease

Hepatic accumulation of fat greater than 5% to 10% by weight is termed non-alcoholic fatty liver disease (NAFLD) (Neuschwander-Tetri & Caldwell, 2003). The term NAFLD encompasses a wide spectrum of liver pathologies. The first stage of NAFLD is simple steatosis, characterized by intracytoplasmic lipid droplets in the hepatocyte. Inflammation and necrosis of hepatocytes marks

the progression to the second stage, non-alcoholic steatohepatitis (NASH). Further inflammatory damage leads to fibrosis, with half of NASH patients progressing to this stage (Sheth et al., 1997). Fifteen percent of patients with fibrosis advance to cirrhosis (Sheth at al, 1997). If not detected and corrected, cirrhosis can cause portal hypertension, hepatocellular carcinoma, and even liver failure (Zafrani, 2004; Luyckx et al., 2000). It is estimated that 3% of patients with NAFLD develop liver failure or require liver transplantation (Sheth et al., 1997).

The current hypothesis for the development for NAFLD involves a two-stage process (Brunt, 2004). In the first stage, reactive oxygen species are generated in hepatocytes from increased levels of TNF-α. TNF-α then initiates various intracellular signals that increase mitochondrial permeability, which subsequently triggers the release of reactive oxygen species (Epstein, 2000). Hepatic lipid accumulation also contributes to the first stage of NAFLD, as compensatory mitochondrial β-oxidation generates more electrons than can be taken into the electron transport chain, creating more mitochondrial reactive oxygen species (Zafrani, 2004). Liver tissue of patients with NASH shows decreased activity of enzymes involved in the electron transport chain, further increasing the formation of reactive oxygen species. This decrease in enzyme activity is correlated with increased serum TNF-α levels (Perez-Carreras et al., 2003). In the second stage, increased oxidative stress from reactive oxygen species triggers the progression from steatosis to steatohepatitis by stimulating

lipid peroxidation, causing cell death (liver cell necrosis) and increased collagen synthesis (fibrosis) (Mehta et al, 2002).

Insulin resistance and an increased supply of FFA to the liver are the two metabolic states most closely correlated with NAFLD (Luyckx et al., 2000). It is estimated that up to 75% of patients with Type 2 diabetes have some form of NAFLD, and past history of Type 2 diabetes is associated with a 26-fold increase in the risk of steatohepatitis (Malnick et al., 2003; Wanless & Lentz, 1990). Obesity is also closely correlated with NAFLD. The risk and severity of hepatic steatosis and steatohepatitis in obese patients is proportional to the degree of obesity (Wanless & Lentz, 1990). In obese children, the prevalence of NAFLD is estimated at approximately 6% to 25% (Vos Louthman et al., 2005).

The underlying insulin resistance that is characteristic of both obesity and Type 2 diabetes may be responsible for the development of hepatic steatosis and NAFLD, rather than the two conditions themselves (Bugianesi et al., 2004). Ninety-eight percent of patients with NASH are insulin resistant, and 87% exhibit attributes of MetS (Angelico et al., 2003). Type 2 diabetic patients with fatty liver have substantially higher insulin resistance than those patients without fatty liver (Kelley et al., 2003). Studies have also shown that insulin resistance, elevated serum TAG levels, and hyperinsulinemia are associated with NAFLD, regardless of body weight and BMI (Marchesini et al., 1999). Although there is strong evidence for an association between obesity, insulin resistance, and NAFLD, non-diabetic and/or normal weight patients with NASH can also exhibit markers of insulin resistance (Comert et al., 2001).

Clinical Features of NAFLD

Most patients with NAFLD present no symptoms. Hepatic steatosis is asymptomatic and usually only detected with biopsy or ultrasonography (Zafrani, 2004). If symptoms are present, they usually resemble those observed in other chronic liver diseases and can include fatigue, malaise, and mild pain in the upper right quadrant. Even without progression to steatohepatitis and fibrosis, hepatic steatosis can influence severity of hepatic insulin resistance (Seppala-Lindroos et al., 2002; den Boer et al., 2004). Seventy-five percent of patients have hepatomegaly; however, in some cases this is difficult to detect due to obesity (Malnick et al., 2003). Slight elevations in serum alanine aminotransferase (ALT) and aspartate aminotransferase are common; however, increases in these liver enzymes can reach up to 15 times the upper limit of normal (Younossi et al., 1999). Contrary to alcoholic fatty liver disease, the aspartate aminotransferase to ALT ratio is usually less than 1, and this ratio increases as cirrhosis develops (Angulo, 1999). Levels of serum alkaline phosphatase and γ-glutamyl transferase are also elevated (Malnick et al., 2003).

Adipocytokines and NAFLD

It is increasingly evident that certain adipocytokines play a role in the pathogenesis of NAFLD, while others can inhibit or reverse it. Increased TNF-α mRNA has been observed in livers of *ob/ob* mice; overproduction of TNF-α in liver tissue is thought to play a key role in the pathogenesis of fatty liver disease in these mice (Tilg & Diehl, 2000). Prevention of excessive hepatic TNF-α

production through infusion of a neutralizing TNF-α antibody significantly improved fatty liver disease in *ob/ob* mice (Li, et al., 2003). Conversely, *ob/ob* mice given adiponectin had significantly reduced plasma levels of TAG and FFA, without affecting plasma insulin and glucose levels (Xu et al., 2003). After 2 weeks of adiponectin treatment, the liver-to-body weight ratios of these mice were significantly reduced, and were associated with decreased hepatic lipid content and decreased serum ALT levels. In humans (adults and children), plasma concentrations of adiponectin are significantly lower in patients with NAFLD compared to both obese and healthy people (Aygun et al., 2006; Pagano et al., 2005; Vos Louthan et al., 2005; Zou et al., 2005). Furthermore, plasma adiponectin concentration is inversely associated with hepatic insulin sensitivity and hepatic lipid content (Bugianesi et al., 2005b). Thus, finding ways to increase adiponectin concentrations while decreasing TNF-α concentration in the body could be a useful component of NAFLD treatment.

Treatment and Management of NAFLD

Currently, there exists no single treatment regimen for patients with NAFLD. Therefore, management is directed towards correction of risk factors and treatments attempting to correct underlying causative conditions. Since insulin resistance is strongly associated with NAFLD, drugs targeting insulin resistance are a potential treatment option. Metformin, a member of the biguanide family of anti-diabetic drugs, has been shown to reduce serum ALT concentrations, improve hepatic insulin sensitivity, and reduce liver size in

NAFLD patients; however, results based on improvements in liver histology are inconsistent (Bugianesi et al., 2005a; Nair et al., 2004; Uygun et al., 2004). In addition, thiazoledinediones have been shown to be effective for improving insulin sensitivity and aminotransferase levels, liver size and fat content, as well as histology (Brunt et al., 2004; Promrat et al., 2004; Sanyal et al., 2004). For all NAFLD patients, exercise and diet are important components of management, and weight must be monitored and controlled. Measures must be taken to improve insulin resistance, and hyperlipidemia must be treated (Zafrani, 2004).

Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) refers to a family of positional and geometrical isomers of the 18-carbon, polyunsaturated fatty acid linoleic acid. The major isomer in foods is the *cis*-9, *trans* -11 (c9,t11) isomer, followed by the *trans*-7, *cis*-9; *cis*-11, *trans*-13; *cis*-8, *trans*-10; and the *trans*-10, *cis*-12 (t10,c12) isomers (Dobson, 2002). Sources of CLA include a number of foods, but the highest concentrations are found in meats and dairy products from ruminant animals. Although there are 28 possible geometric isomers of CLA, most research has been directed toward the two isomers showing the highest biological activity: the c9,t11 and the t10,c12 isomers (Figure 1) (Belury, 2002; Pariza et al., 2001).

Biosynthesis of CLA can occur via ruminal biohydrogenation of linoleic or linolenic acid, or via endogenous synthesis in mammary glands or adipose tissue (Figure 2). The c9,t11 CLA isomer is the first intermediate product in the

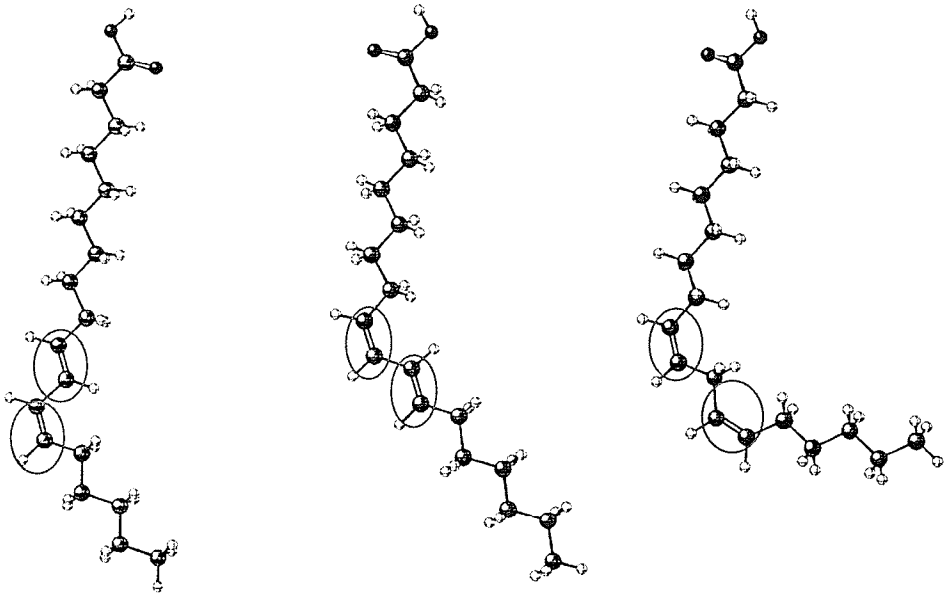


Figure 1. Structure of selected CLA isomers. From left to right: c9,t11CLA, t10,c12 CLA, linoleic acid. Double bonds are circled. Adapted from Bauman et al. (1999).

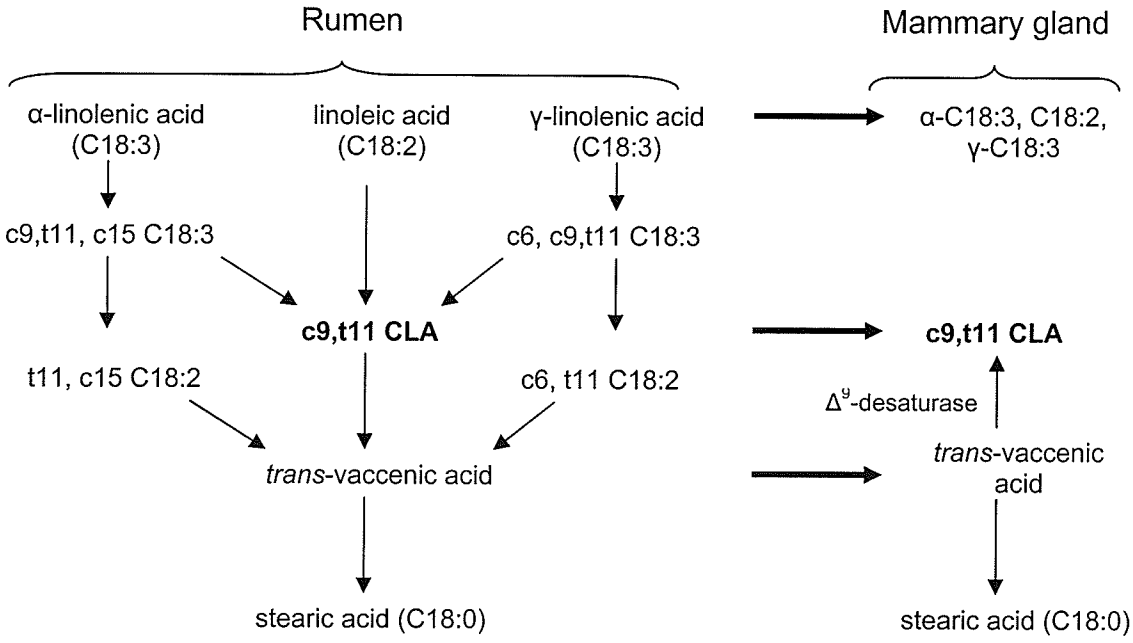


Figure 2. Pathways involved in ruminal biohydrogenation and endogenous synthesis of CLA. Adapted from Dihman et al. (2005).

biohydrogenation of linoleic acid by the enzyme linoleate isomerase, which is produced by the microorganism *Butyrivibrio fibrisolvens* and other bacterial species (Kepler et al., 1967). A portion of the c9,t11 isomer is rapidly reduced to *trans*-vaccenic acid or stearic acid, becoming available for absorption in the small intestine. However, a portion of the c9,t11 isomer produced by isomerization of linoleic acid escapes complete biohydrogenation, is absorbed from the intestine and incorporated into milk fat. It was originally assumed that the rumen was the primary site of origin of c9,t11 CLA in milk fat. Recently, however, it has been suggested that only a small portion of c9,t11 CLA escapes biohydrogenation in the rumen, and that the major portion of c9,t11 CLA in milk comes from endogenous synthesis in the mammary gland via a pathway involving the desaturation of *trans*-vaccenic acid by the $^{\Delta 9}$-desaturase enzyme (Yang, 1999; Griinari et al., 2000; Corl et al., 2001). The t10,t12 CLA content of ruminant products is solely from absorption in the gastrointestinal tract. The pathways for synthesis of the other CLA isomers naturally found in milk fat are still unknown, but is likely from bacterial action in the rumen (Belury, 2002). The CLA content of ruminant milk and meat is variable and dependent on a number of factors, including diet, age, and breed of the animal (Dihman et al., 2005).

Endogenous synthesis of CLA from vaccenic acid has also been observed in humans, rats, and mice (Turpeinen et al., 2002; Corl et al., 2003; Santora et al., 2000). CLA can also be produced synthetically, using a base catalyzed reaction with linoleate as a substrate, with the majority of isomers produced being in the c9,t11 and t10,c12 forms, although varying amounts of the t8, c10

and c11, t13 isomers and other positional isomers are also produced (Pariza et al., 2001; Dobson, 2002).

The first evidence of a potential role for CLA in disease prevention appeared in 1987, when synthetically-produced CLA was shown to inhibit carcinogenesis in mice (Ha et al., 1987). Since then, numerous beneficial physiological effects of CLA have been observed, including reduced adiposity in numerous animal models, modulation of immune function and prevention and management of arteriosclerosis, carcinogenesis, and Type 2 diabetes (Park et al., 1997; Sisk et al., 2001; Sugano et al., 1998; Wilson et al., 2000; Ip et al., 1996; Ip et al., 1991; Houseknecht et al., 1998).

Beneficial effects of CLA have been demonstrated in humans. The most promising effects are related to weight loss, improvement in body composition, improvement in glucose metabolism and insulin resistance, as well as favourable effects on the lipid profile (Risérus et al., 2004; Salas-Salvadó et al., 2006). However, the use of CLA as a nutraceutical is controversial, as some human studies have shown negative effects such as worsening of insulin resistance, elevated VLDL cholesterol, lowering of high-density lipoprotein (HDL) cholesterol, and elevated plasma markers of inflammation (Risérus et al., 2002a; Whigham et al., 2004; Tricon et al., 2004; Smedman & Vessby, 2001; Moloney et al., 2004).

CLA and adipocytokines

Research has a shown a connection between dietary CLA and adipocytokines. Male Sprague Dawley rats fed a CLA mixture (1.5% wt/wt) for 3

weeks had significantly lower serum TNF-α concentrations (Yamasaki et al., 2003). Results have been similar in mice, as 8 weeks of feeding a 1% (wt/wt) dietary CLA mixture decreased serum concentrations of TNF-α (Akahoshi et al., 2002). However, effects of CLA on TNF-α production in the liver have not been reported.

CLA has also been shown to affect the production of adiponectin. As will be discussed in more detail in the following section, CLA is a peroxisome proliferator-activated receptor agonist. The human adiponectin promoter contains a functional peroxisome proliferator response element; therefore, CLA may increase transcription of the adiponectin gene by this mechanism, thereby increasing plasma concentrations of adiponectin (Iwaki et al., 2003). This hypothesis is supported by a study performed by Nagao et al. (2003b). Six-week-old male Zucker diabetic fatty (ZDF) rats fed 1% (wt/wt) CLA mixture for 8 weeks had approximately 40% more plasma adiponectin compared to control ZDF rats. Noto et al. (2004) found serum adiponectin concentrations were 26% higher in male *fa/fa* Zucker rats fed a 1.5% (wt/wt) CLA mixture for 8 weeks, compared to *fa/fa* control rats. Higher fasting serum adiponectin concentrations were also observed in male *fa/fa* Zucker rats fed a CLA mixture for 8 weeks, compared to rats consuming a control diet (Noto, 2004). This elevation in serum adiponectin was concurrent with improved oral glucose tolerance. Modification of cytokine production (decreased TNF-α and increased adiponectin) by CLA could contribute to improved insulin action in the liver.

CLA and Lipid Metabolism

Peroxisome Proliferator Activated Receptor α

CLA influences transcription and expression of a number of genes by acting as a ligand for a group of membrane-bound nuclear transcription factors known as peroxisome proliferator activated receptors (PPARs) (Moya-Camarena et al., 1999; Peters et al., 2001). PPARs directly modulate gene activity by controlling transcription for genes involved in lipid metabolism. In the nucleus, PPARs combine with retenoic acid receptor (RXR) to form a functional heterodimer, but transcriptional activation is inhibited due to association of the heterodimer with co-repressors (Wahli, 2002; Smith, 2003). Ligand binding causes release of co-repressors, producing a ligand-activated PPAR:RXR complex. This activated PPAR:RXR complex is able to bind to a specific sequence of nucleotides [the peroxisome proliferator response element (PPRE)], which causes a change in chromatin structure that allows transcription to begin (Desvergne & Wahli, 1999).

To date, three PPAR isotypes have been identified: PPARα, PPARβ, and PPARγ. PPARβ (also known as PPARδ) is expressed in all tissues (Ferré, 2004). Less is known about the physiologic function of PPARβ compared to the other isoforms, however, recent studies have identified the role of PPARβ in increased reverse cholesterol transport in macrophages, fibroblasts, and intestinal cells, as well as modification of muscle fibre type (Oliver et al., 2001; Evans, 2004). PPARγ is involved in adipocyte differentiation as well as lipid anabolism; direct target genes of PPARγ include those coding for adipocyte fatty acid binding

protein and lipoprotein lipase (Debril et al., 2001). Thiazoledinediones exert their insulin-sensitizing properties by acting as a ligand for PPARγ (Lehmann et al., 1995; Wilson et al., 1996; Berger et al., 1996). Some studies have shown that CLA may also act as a PPARγ ligand, thus explaining the insulin-sensitizing effects of CLA (Houseknecht et al., 1998; Peters et al., 2001).

In contrast, PPARα is highly expressed in liver tissue and its target genes participate in lipid catabolism [which include hepatic fatty acid binding protein, acyl-CoA synthase (required for activation of fatty acids for subsequent oxidation), stearoyl-CoA desaturase 1 (also known as $^{\Delta 9}$desaturase; desaturates long chain saturated fatty acids), acyl-CoA oxidase (ACO; the rate limiting enzyme in peroxisomal β-oxidation), carnitine palmitoyl transferase 1 (CPT1; required for transport of fatty acids across the mitochondrial membrane), and hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase; required for ketone body formation) (Desvergne & Wahli, 1999; Wahli, 2002; Ferré, 2004)]. Polyunsaturated fatty acids (PUFAs) are known PPAR ligands, and CLA (a PUFA) has been shown to be a strong ligand and activator of PPARα (Forman et al., 1997; Krey et al., 1997; Moya-Camarena et al., 1999). Hypolipidemic drugs known as fibrates, which are often prescribed to patients with Type 2 diabetes to help normalize lipid levels, exert their effects by acting as PPARα ligands (Ferré, 2004). It is possible that by acting as a ligand for PPARα, CLA may help decrease hepatic steatosis by increasing transcription of genes involved in hepatic lipid catabolism.

Although it is likely that CLA controls hepatic lipid accumulation by activation of PPARα, studies with PPARα knock-out mice provide strong evidence that PUFA-dependent control of hepatic lipid metabolism acts via two separate pathways: one requiring PPARα for regulation of peroxisomal and microsomal oxidation enzymes, and one independent of PPARα involving PUFA-mediated suppression of lipogenic gene expression (Ren et al., 1997; Xu et al., 1999).

Sterol Regulatory Element-Binding Proteins

Sterol regulatory element-binding proteins (SREBPs), another class of transcription factors, are transmembrane proteins of 125 kD, oriented in the bilayer of the endoplasmic reticulum as a helical hairpin, which must undergo two proteolytic cleavages to produce the active 68 kD fragment (Brown et al., 2000). There are three identified SREBP isoforms known as SREBP-1a, SREBP-1c, and SREBP-2 (Sakai & Rawson, 2001). Livers from adult animals predominantly synthesize SREBP-1c and SREBP-2 (Shimano et al., 1997). SREBP target genes include enzymes of cholesterol biosynthesis: HMG-CoA reductase, HMG-CoA synthase, and squalene synthase. SREBPs also bind via a helix-loop-helix domain to regulatory sequences in the promoters of genes involved in the biosynthesis of fatty acids, such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase, as well as glycerol 3-phosphate acyltransferase, an enzyme involved in the production of TAG (Horton, 2002). All three SREBPs are capable of activating each of the known target genes,

although with varying efficiencies. Data from studies using transgenic mice suggest that the SREBP-1c isoform is more selective in activating fatty acid biosynthesis genes, while SREBP-2 is more specific for controlling cholesterol biosynthesis (Shimano et al., 1996; Shimano et al., 1997; Horton et al., 1998). It has also been suggested that SREBP-1 is crucial for nutritional induction of hepatic lipogenic enzymes (Shimano et al., 1999).

Increased levels of nuclear SREBP-1c were found in the fatty livers of *ob/ob* and aP2-SREBP-1c mice and were associated with increased mRNA expression of SREBP target genes involved in fatty acid biosynthesis (Shimomura et al., 1999). Similarly, the fatty liver normally present in *ob/ob* mice was attenuated with an SREBP-1 deficiency (although obesity and insulin resistance persisted), which correlated with decreased hepatic mRNA for SREBP-1 target enzymes (Yahagi et al., 2002). These results suggest that increased levels of nuclear SREBP-1c contribute to the elevated rates of hepatic fatty acid synthesis and the resulting steatosis in obese mice.

In vitro studies have shown that PUFAs can suppress both expression and activity of SREBP-1 (Xu et al., 2001; Yoshikawa et al., 2002). Male Sprague Dawley rats fed a PUFA-supplemented diet had 50% and 42% lower hepatic concentrations of mature nuclear SREBP-1 and SREBP-2 proteins, respectively, accompanied by 63% and 52% reductions in hepatic expression of the SREBP-1 and SREBP-2 target genes FAS and HMG-CoA synthase (Xu et al., 2001). In this study, PUFA supplementation did not affect levels of the membrane-bound precursor forms of SREBP-1 or SREBP-2. To examine the effect of dietary

PUFA on hepatic TAG deposition, Sekiya et al. (2003) fed *ob/ob* mice PUFAs for 7 days, and found reductions in the mature form of SREBP-1 protein and also reduced expression of lipogenic genes such as FAS and steroyl-CoA desaturase-1 in the liver. Once again, PUFA supplementation did not affect protein levels of membrane-bound precursor SREBP-1, or hepatic mRNA expression. Interestingly, hepatic TAG content and plasma ALT levels were improved with PUFA administration, similar to the effect of PPARα activators. By inference, CLA (a PUFA) may ameliorate hepatic steatosis both by activation of PPARα (increased lipid catabolism) and by suppression of SREBPs (reduced lipid anabolism).

Interestingly, SREBPs may also affect hepatic insulin signaling as well as hepatic lipid synthesis. As stated above, SREBP-1c is highly expressed in liver tissue of *ob/ob* mice, and contributes to the development of hepatic steatosis. Overproduction of the nuclear active form of SREBP-1c is associated with a marked reduction in hepatic IRS-2 mRNA levels (Ide et al., 2004). IRS-2 mRNA is elevated in the livers of SREBP-1-deficient mice, compared with those of wild-type mice both at fasting and re-feeding (Ide et al., 2004). Increased IRS-2 mRNA levels have also been detected in SREBP-1c-knockout mice (Liang et al., 2002). Together, these data provide strong evidence of an inverse relationship between hepatic SREBP-1c and IRS-2 expression.

CLA, Insulin Sensitivity, and Hepatic Steatosis

Results from studies examining the effects of CLA on animal models of obesity, insulin resistance, and Type 2 diabetes have been contradictory and appear to be species specific.

Six-week old male ZDF rats fed 1.5% (wt/wt) CLA (containing approximately the same amount of c9,t11 and t10,c12 CLA) for 2 weeks had lower plasma insulin concentrations when compared to control rats (Houseknecht et al., 1998). CLA-fed ZDF rats had plasma insulin concentrations similar to ZDF rats fed troglitazone, a PPARγ-activating thiazoledinedione. Glucose tolerance was also improved in the CLA-fed ZDF rats, once again, in a manner similar to the troglitazone-fed ZDF rats. Interestingly, the CLA diet decreased circulating FFA in ZDF rats compared to lean controls.

Ryder et al. (2001) also observed beneficial effects of CLA-feeding in ZDF rats. Compared to controls, 6 week-old rats fed 1.5% (wt/wt) CLA as a 50:50 mixture of the c9,t11 and t10,c12 isomers, for 14 days, had reduced adiposity, fasting glucose, plasma insulin, and FFA, as well as improved glucose tolerance and insulin sensitivity in muscle. These beneficial effects were not observed upon treatment with the c9,t11 CLA isomer alone. Both CLA diets significantly reduced plasma TAG. Muscle and adipose expression of uncoupling protein-2, a regulator of fatty acid oxidation, was up-regulated in the 50:50 CLA treatment, and further up-regulated with the c9,t11 isomer alone, suggesting a role for CLA (and specifically c9,t11 CLA) in increasing fatty acid oxidation. Unfortunately, this study and the previously discussed study did not examine the effects of CLA

on hepatic insulin signaling, hepatic steatosis, or hepatic fatty acid oxidation. In addition, these studies only examined short-term effects of CLA feeding.

Noto et al. (2006) examined the effects of CLA feeding in the *fa/fa* Zucker rat, a model of impaired glucose tolerance and hyperinsulinemia (but not hyperglycemia). Six-week old lean and *fa/fa* male Zucker rats were fed a diet supplemented with a 1.5% (wt/wt) CLA mixture or a control diet containing soybean oil. After 8 weeks of feeding, CLA–fed *fa/fa* rats showed improved oral glucose tolerance compared to control-fed *fa/fa* rats. This study also examined the effects of CLA on hepatic steatosis, and found that the CLA diet reduced liver weight and lowered hepatic TAG accumulation compared to the control diet. On a molecular level, *fa/fa* rats fed CLA had lower hepatic ACC and higher ACO mRNA expression than *fa/fa* controls, suggesting that CLA decreases hepatic steatosis by inducing fatty acid oxidation (possibly through activation of PPARα) while reducing fatty acid synthesis (possibly through suppression of SREBP-1, although SREBP-1 mRNA expression or protein levels were not reported). This study did not investigate whether one specific CLA isomer was responsible for the observed effects.

Contrary to the beneficial effects observed upon CLA feeding in rat models, CLA appears to produce insulin resistance and fatty liver in murine models of obesity and insulin resistance (Hargrave et al., 2003). Using insulin tolerance testing, Tsuboyama-Kasaoka et al. (2000) found that female C57BL/6J mice fed a CLA mixture (1% wt/wt) displayed higher insulin resistance, as well as elevated fasting and feeding insulin levels compared to controls. Livers of CLA-

fed mice were larger, heavier, and pale in colour compared to those of control mice, indicating hepatic TAG accumulation. Prolonged consumption of the CLA diet led to lower adipose mRNA levels of FAS and ACO. The authors did not investigate the effects of CLA feeding on hepatic expression of lipid metabolizing enzymes, or its effects on hepatic insulin sensitivity.

To gain insight into the isomer-specific effects of CLA, Clément et al. (2002) fed female C57BL-6J mice a control diet, a diet containing linoleic acid, a diet containing purified c9,t11 CLA, or a diet containing purified t10,c12 CLA for 4 weeks. Mice fed t10,c12 CLA were markedly hyperinsulinemic compared to the control, linoleic acid-fed, and c9,t11-fed mice. Hepatomegaly and intracellular hepatic accumulation of lipid was observed only in mice fed the t10,c12 diet. Supplementation with t10,c12 CLA, but not linoleic acid or c9,t11 CLA, slightly (but significantly) raised SREBP-1a mRNA, while none of the diets influenced mRNA levels of SREBP-1c or SREBP-2. SREBP protein levels were not measured. Interestingly, hepatic expression of PEPCK was significantly lower after treatment with t10,c12 CLA, suggesting that the t10,c12 isomer increases hepatic insulin sensitivity in this model.

Roche et al. (2002) also found isomer-specific effects of CLA in *ob/ob* mice. Six-week-old male *ob/ob* mice fed a diet containing the c9,t11 isomer (4.43% wt/wt) for 4 weeks had significantly reduced serum TAG and FFA compared to mice fed the t10,c12 isomer (4.59% wt/wt) or control diet. Mice fed t10,c12 CLA became severely insulin resistant with markedly higher serum glucose and serum insulin (but no significant rise in serum TAG or FFA). In the

liver, the c9,t11 CLA treatment reduced hepatic SREBP-1c mRNA expression as well as decreased levels of the precursor and mature SREBP-1c proteins. The t10,c12 isomer had no effect on these parameters, suggesting that the c9,t11 isomer may ameliorate hepatic steatosis via suppression of both SREBP-1c mRNA and protein levels. In addition, these researchers also found that c9,t11 CLA significantly reduced TNF-α expression in the liver, possibly leading to increased hepatic insulin sensitivity. Unfortunately, this study did not examine if the decrease in hepatic SREBP-1c mRNA and protein levels correlated with levels of SREBP-1c downstream enzymes, nor did it report if and how the decrease in SREBP-1c corresponded to decreases in hepatic lipid accumulation.

Takahashi et al. (2003) examined the effects of a CLA mixture on both expression and activity of lipid metabolizing enzymes. Compared to control, linoleic acid, and palmitic acid–supplemented diets, 1.5% (wt/wt) dietary CLA (approximately 46.2% c9,t11 CLA, 48.1% t10,c12 CLA, and 5% other isomers) for 3 weeks markedly increased liver weights, hepatic TAG levels, and hepatic cholesterol content in 5-week old C57BL/6J and ICR mice. On a molecular level, livers of CLA –fed mice showed higher activity as well as higher levels of ACC and FAS mRNA, suggesting increased activation of SREBP-1. Hepatic SREBP-1 mRNA was also higher in mice fed the CLA diet. Interestingly, activity and expression of enzymes involved in hepatic fatty acid oxidation, such as ACO and CPT, were also significantly higher. Simultaneous induction of fatty acid oxidation and fatty acid synthesis could not be explained; although it is possible that CLA both activated PPARα and up-regulated SREBP-1 in this experiment.

Overall, this study demonstrated that CLA up-regulates not only production, but also the activity of various enzymes involved in fatty acid synthesis and oxidation. The magnitude of the increase in enzyme activity in this study could not be explained solely by elevated mRNA levels, suggesting that CLA imparts some of its beneficial effects through another mechanism.

Additional Mechanisms of Action for CLA

AMP-Activated Protein Kinase

Although CLA may directly affect fatty acid synthesis and oxidation by influencing gene transcription, presumably by suppressing SREBP or activating PPARα, it may also exert indirect effects on glucose and lipid metabolism. AMP-activated protein kinase (AMPK) is a sensor of cellular energy status present in all eukaryotic cells that responds to changes in the intracellular AMP:ATP ratio (Carling, 2004). It is a heterotrimeric protein consisting of a catalytic α and regulatory β and γ subunits (Long & Zierath, 2006). Increases in the AMP:ATP ratio trigger AMPK to inhibit anabolic pathways that consume ATP and stimulate catabolic pathways that generate ATP. In order to be activated, AMPK must be phosphorylated on threonine^{172} within the α catalytic subunit by an upstream protein kinase known as LKB1 (Woods et al., 2003). Additionally, calmodulin-dependent protein kinase kinase can also phosphorylate AMPK; however, activation by this enzyme is dependent on increases in intracellular calcium ions and occurs independently of the AMP:ATP ratio (Hurley et al., 2005). Activated AMPK inhibits lipid biosynthesis by phosphorylating (and consequent

inactivation) ACC1, glycerol phosphate acyltransferase, and HMG-CoA reductase (Henin et al., 1995; Winder & Hardie, 1999; Muoio et al., 1999; Hardie et al., 2003). Although several protein kinases can phosphorylate ACC, current evidence supports the hypothesis that ACC phosphorylation in intact hepatocytes and in the liver in vivo is mainly carried out by activated AMPK (Hardie et al., 2003).

The product of ACC, malonyl-CoA, is both a precursor for the biosynthesis of fatty acids and a potent inhibitor of β-oxidation because it prevents transport of long-chain fatty acids into the mitochondrial matrix through allosteric inhibition of CPT1. Thus, when ACC is inactivated, cellular concentrations of malonyl-CoA decrease, resulting in less inhibition of CPT1 and increased fatty acid oxidation (Muoio et al., 1999). In this manner, levels of malonyl-CoA have been shown to regulate intracellular fatty acid oxidation in a variety of tissues, including the liver (McGarry & Brown, 1997; Ruderman et al., 1999). ACC exists in two isoforms. The main role of ACC1 is to synthesize the malonyl-CoA used for de novo fatty acid synthesis, and ACC2 is thought to produce the malonyl-CoA that inhibits CPT1 (Abu-Elheiga et al., 2001). Although the two ACC isoforms are expressed in different tissues, the liver contains both isoforms of ACC (Ruderman et al., 2003).

In addition to inactivating anabolic enzymes, AMPK influences expression of genes coding for enzymes involved fatty acid biosynthesis (ACC1 and FAS) (Woods et al., 2000). AMPK activation also suppresses the expression of SREBP-1c, thereby decreasing expression of its downstream lipogenic enzymes

(Zhou et al., 2001). The fact that AMPK inhibits lipid biosynthesis both acutely (by phosphorylation of ACC1, glycerol phosphate acyltransferase, and HMG-CoA reductase) and chronically (by reduced expression of ACC-1, FAS, SREBP-1c) emphasizes the importance of AMPK in lipid metabolism.

AMPK also influences glucose metabolism through its effects on gene expression. Activation of AMPK in liver cells has been shown to decrease expression of PEPCK and glc 6-Pase, both in vitro and in vivo (Lochhead et al., 2000; Yamauchi et al., 2002). As elevated gluconeogenesis is a major contributor to hyperglycemia in insulin resistance, therapeutic agents that target activation of AMPK might be beneficial for glycemic control in insulin resistance. In vitro studies of isolated hepatocytes and hepatoma cells have shown that 5-aminoimidazole-4-carboxamide ribofuranoside (AICAR), an activator of AMPK, reduces hepatic gluconeogenesis (Lochhead et al., 2000). Male Zucker *fa/fa* rats given an acute dose of AICAR for 90 minutes had a greater than 50% decrease in endogenous glucose production (Bergeron et al., 2001). Similarly, Iglesias et al. (2002) found that acute administration of AICAR to rats resulted in decreased hepatic glucose output and decreased liver TAG content, leading them to hypothesize that these beneficial effects of AMPK activation are achieved by bypassing the defective insulin signaling pathway or by creating metabolic conditions that improve insulin action. Exposure to AICAR for 7 weeks decreased plasma TAG, FFA, fasting plasma insulin and glucose, as well as improved oral glucose tolerance in male *fa/fa* Zucker rats (Buhl et al., 2002).

Since AMPK has the ability to increase hepatic insulin sensitivity and decrease hepatic glucose output by controlling transcription for gluconeogenic enzymes, and since administration of adiponectin to mice has been shown to decrease expression of PEPCK and glc 6-Pase, it is possible that adiponectin exerts its insulin sensitizing effects through activation of AMPK. Using isolated hepatocytes, Yamauchi et al. (2002) showed that full-length adiponectin stimulates phosphorylation and activation of AMPK, and that ACC phosphorylation in response to adiponectin treatment of hepatocytes requires AMPK. Globular adiponectin did not activate hepatic AMPK in this study, even at higher doses. In addition, adiponectin elevated ACC phosphorylation in the livers of mice. Adiponectin treatment lowered expression of PEPCK and glc 6-P in the liver. Furthermore, blocking AMPK activity in the liver increased expression of these gluconeogenic enzymes, suggesting that activation of AMPK in the liver is necessary for adiponectin to reduce expression of these genes.

CLA may exert its beneficial effects on liver lipid metabolism and hepatic glucose production by increasing plasma concentrations of adiponectin with subsequent AMPK activation. As mentioned before, the human adiponectin promoter contains a PPRE, which implies that CLA may increase transcription of the adiponectin gene, thereby increasing plasma levels of adiponectin (Iwaki et al., 2003). This hypothesis is supported by a study performed by Nagao et al. (2003b). Compared to control rats, six-week old male ZDF rats fed a 1% (wt/wt) CLA mixture for 8 weeks showed higher plasma adiponectin concentrations, accompanied by a 65% reduction of plasma insulin and glucose concentrations.

Similarly, higher fasting serum adiponectin concentrations, lower fasting serum insulin concentrations, and improved oral glucose tolerance were observed in male *fa/fa* Zucker rats fed a CLA mixture for 8 weeks, compared to rats consuming a control diet (Noto, 2004). While these studies demonstrate the effects of CLA on circulating adiponectin concentrations and insulin resistance, they did not directly measure the effects of the CLA-mediated increase of plasma adiponectin on AMPK activation, or the isomer-specific effects of CLA on markers of AMPK activation.

STUDY RATIONALE

Obesity in developed countries is an emerging epidemic and is associated with a number of health complications, including insulin resistance and MetS, the later of which is a risk factor for development of Type 2 diabetes and cardiovascular disease. In 2000, the estimated cost of obesity and MetS in North America was $117 billion (Pierce, 2003). The continual rise in obesity rates predicts a parallel increase in the prevalence of MetS, which will impose a heavier burden on North America's health care systems. The discovery of dietary components that aid in the treatment and/or prevention of the complications associated with MetS could have an important impact not only on the cost of health care, but also the health and quality of life of patients.

Previous research suggests that CLA has potential ameliorative effects for MetS. Insulin resistance and glucose intolerance are attenuated by CLA-feeding, evidenced by reduced fasting hyperinsulinemia and improved response to glucose doses (Houseknecht et al., 1998; Ryder et al., 2001; Noto, 2004). Lipidemia is also improved, reflected by reduced fasting serum cholesterol, FFA, and TAG (Houseknecht et al., 1998; Ryder et al., 2001; Noto et al., 2006).

Before the start of this project, little information was available regarding isomer-specific effects of CLA on hepatic steatosis. It had been observed that liver lipid concentration was reduced by 62% after 8 weeks of feeding a 1.5% (wt/wt) CLA mixture in 6-week-old *fa/fa* Zucker rats (Noto et al., 2006). However, CLA mixtures contain numerous CLA isomers, and it was unknown if the lipid-

lowering effects of CLA in the liver of *fa/fa* Zucker rats (and other rat models) were due to the actions of a single isomer or synergistic actions of several isomers. In mice, it was known that t10,c12 CLA induced lipid accumulation in the liver, while c9,t11 CLA did not (Clément et al., 2001; Roche et al., 2002). However, while studies in mice demonstrated a negative effect of t10,c12 CLA on insulin sensitivity, t10,c12 CLA improved insulin sensitivity in rat models. Therefore, it seemed relevant to study isomer-specific effects of CLA on liver lipid accumulation in a rat model.

Discovering the molecular mechanisms that contribute to reduced hepatic steatosis was also a goal of the current project. Based on previous research conducted in our laboratory, reduced hepatic steatosis observed with CLA-feeding was accompanied with higher levels of liver fatty acid binding protein and a trend toward higher levels of ACO mRNA, suggesting that CLA elevates lipid oxidation via increased PPARα activation (Noto et al., 2006). While this may be true, a possible inhibitory effect of CLA on hepatic lipogenesis cannot be discounted.

SREBP-1 is a transcription factor involved in transcription of lipid anabolic enzymes. To be activated, the full-length SREBP-1 protein undergoes proteolytic cleavage to yield a smaller fragment SREBP-1 protein that translocates to the nucleus for initiation of transcription. In mice fed CLA, reduced liver lipid accumulation observed with feeding the c9, t11 CLA isomer is associated with reduced expression and activation of SREBP-1 (Roche et al., 2002). How

dietary CLA isomers affect hepatic SREBP-1 activation in rats has never been determined.

In addition to quantity of enzyme present, enzyme activity is a possible target of dietary interventions. AMPK regulates lipid metabolism by phopshorylation and inactivation of several key enzymes involved in lipid metabolism. In order for this to occur, AMPK itself must be activated by phosphorylation. Adiponectin is a known activator of AMPK, and CLA has been shown to elevate plasma concentrations of adiponectin; therefore CLA may indirectly activate AMPK and inhibit lipid synthesis (Yamauchi et al., 2002; Nagao et al., 2003b; Noto, 2004). This project would be the first to study the effect of dietary CLA isomers on AMPK activation in the livers of obese, insulin-resistant rats.

NAFLD is associated with a decrease in liver function as early as the first stage of hepatic steatosis. Reduced hepatic steatosis associated with dietary CLA has also been shown to improve liver function, as seen with improved serum levels of enzymes associated with liver function, such as ALT and alkaline phosphatase (Noto et al., 2006). Therefore, it is important to confirm that the same CLA isomer that reduces hepatic steatosis is also responsible for the improvement in liver function.

It is becoming increasingly evident that inflammation is also a component of MetS (Ridker et al., 2004; Gonzalez et al., 2006; Haffner, 2006). Little information is available on how dietary CLA affects markers of acute-phase inflammation; however, Noto (2004) did discover that a dietary CLA mixture

reduced serum haptoglobin concentration in *fa/fa* Zucker rats fed for 8 weeks. This study will follow-up on that research to reveal the isomer responsible for this reduction in inflammation.

The *fa/fa* Zucker rat is used often as an animal model for obesity and insulin resistance. A single nucleotide substitution in the leptin receptor gene produces an allelic variant referred to as the *fa* gene (Chua et al., 1996). This *fa* gene produces a dysfunctional leptin receptor with reduced binding affinity for leptin (White et al., 1997). Therefore, Zucker rats homozygous for the *fa* gene lack functional leptin receptors and, because of the role of leptin in satiety, become hyperphagic (Kava et al., 1990). In addition to reduced leptin-binding affinity, the leptin receptor the of *fa/fa* Zucker rat exhibits reduced signal transduction capabilities (Yamashita et al., 1997). Obesity in this animal model can present as early as 3 to 5 weeks of age (Kava et al., 1990; Phillips et al., 1996). Hyperinsulinemia and impaired oral glucose tolerance are also observed in this model (Ionescu et al., 1985; Kava et al., 1989). In addition, the *fa/fa* Zucker rat exhibits severe hepatic as well as peripheral insulin resistance (Terretaz et al, 1986a; Terretaz et al., 1986b). Hepatic steatosis and hyperlipidemia present as early as 2 to 4 weeks of age in these animals (Krief & Bazin, 1991). These rats have been shown to also exhibit hypertension (Kurtz et al., 1989). Because it displays many of the characteristics of MetS, the *fa/fa* Zucker rat is an appropriate model for the purposes of this project.

Other rat models of obesity exist, and include both genetically-caused and diet-induced obesity. The Jcr:LA-*cp* rat is often used as a model for obesity. This particular rat develops obesity due the presence of the *cp* gene, which translates to a stop codon in the extracellular domain of the leptin receptor and leads to an absence of leptin receptors on plasma membranes (Russell, 1995). This rat model displays hyperlipidemia (mainly due to increased plasma VLDL and HDL), hyperinsulinemia, and impaired glucose tolerance. Unlike the *fa/fa* Zucker rat, the Jcr:LA-*cp* rat develops vascular and myocardial lesions, making it an appropriate model in which to study atherosclerosis (Russell & Koeslag, 1990). However, as the effects of CLA on the atherosclerotic process was not one of our main outcomes, it was not necessary to use the Jcr:LA-*cp* rat.

Inducing obesity in rats by means of dietary manipulation is another method of examining obesity in the rat. The most commonly-used diet-induced model is the Sprague Dawley rat. Obesity in this rat model can be induced by high-fat feeding, high-energy feeding (moderately high in fat and sugar) or palatable liquid diets (Mercer & Archer, 2005). However, some Sprague Dawley rats do not develop obesity when presented with high-fat or high-energy diets, and it is impossible to differentiate between those rats that will develop obesity and those that will not. Furthermore, development of diet-induced obesity in these rats can take a long time and may take longer in some rats than in others. Diet-induced obese Sprague Dawley rats have significantly higher fasting plasma glucose, suggesting that these animals are diabetic (Lauterio et al., 1994); however, the aim of this project was to assess the effects of CLA in MetS, which

is not necessarily characterized by hyperglycemia and the diabetic state. In addition, at this point the author is unaware of studies that have examined how diet-induced obesity affects hepatic lipid content. For the above mentioned reasons, we concluded that using that *fa/fa* Zucker rat would be the most appropriate model for use in this project.

The current study will provide insight into the isomer-specific effects of CLA in MetS, and will provide more information on how hepatic lipid metabolism is affected by dietary CLA isomers. Purified c9,t11 and t10,c12 CLA isomers (93% and 92% pure, respectively) will be fed separately to compare directly the actions of these two isomers. Commercial mixtures of CLA isomers have been used extensively in previous CLA studies; therefore, we will also examine the effects of two commercial CLA mixtures, one containing two CLA isomers (predominantly c9,t11 and t10,c12 CLA) and the other containing 4 CLA isomers. Since the 2-isomer CLA mixture also contains small amounts of other CLA isomers, we will also look at the effects of a diet containing equal amounts of the c9,t11 and t10,c12 purified isomers that we combine in our laboratory. In addition, the 4-isomer CLA mixture contains significant amounts of the t8, c10 and c11, t13 isomers, which may enhance the beneficial effects of dietary CLA. These five CLA diets will allow us to clarify further if the effects observed are due to the actions of one isomer, or by a synergistic effect of multiple isomers.

Diets will be formulated to contain 0.4% (wt/wt) of either c9,t11 or t10,c12 CLA, or 0.4% c9, t11 CLA ***and*** 0.4% t10,c12 CLA (for dietary treatments where

isomers will be fed in combination). This level of CLA was chosen based on past experiments in our laboratory where hepatic lipid-lowering was observed with this dose of CLA (Noto et al., 2006). The study duration of 8 weeks was chosen also based on this past experiment. Eight weeks is considered a long-term feeding study in lipid metabolism, and thus will allow us to assess the long-term effects of dietary CLA. A sample size of 10 per each dietary group was chosen based on the calculated sample size required to produce a significant difference in serum adiponectin concentration, with a power of 0.80 and α of 0.05 (Zirk, 2005).

This project was separated into two 8-week feeding studies. The purpose of Study 1 was to assess the effect of dietary CLA on conditions associated with MetS, including glycemia and lipidemia, which are measured in the fasted state. Hepatic lipids, serum ALT, serum haptoglobin, and hepatic proteins involved in lipid metabolism were also measured in the fasted state. In addition, a group of lean and *fa/fa* Zucker rats was included to measure differences in some of the above mentioned parameters at 6 weeks of age. These baseline rats were also used to assess the extent of change in measured parameters after the 8 week feeding trial relative to baseline values (i.e. is the magnitude of change elicited by CLA enough to restore parameters to baseline values?).

For a more complete picture of how CLA potentially regulates hepatic lipid metabolism, a second 8-week feeding study was conducted to measure the effects of dietary CLA isomers on hepatic proteins involved in lipid metabolism, this time in the fed state. Hepatic lipid metabolism is influenced by nutritional

state (i.e. fed vs. fasted). In fasted conditions, hepatic metabolism favours lipid oxidation for energy production. Situations where dietary energy is readily available (fed state) are characterized by lipid storage. Thus, the degree of lipid synthesis and breakdown varies from the fasted to fed state. However, lipid oxidation still may be occurring in the fed state, and may be influenced by the type of dietary lipid. For example, Sprague Dawley rats fed a meal containing PUFAs had less inactivation of AMPK in the fed state accompanied by higher levels of inactive ACC, indicating higher lipid oxidation, compared to rats fed a control meal (Suchancova et al., 2005). This finding substantiates the need to measure the effect of CLA on hepatic lipid metabolism in both the fed and fasted states.

HYPOTHESES AND OBJECTIVES

Study 1:

It was hypothesized that:

1. The t10,c12 CLA isomer will favourably modify hepatic steatosis, liver dysfunction and inflammation associated with MetS.

2. The t10,c12 CLA isomer reduces hepatic steatosis by a mechanism involving indirect suppression of SREBP-1 activation and indirect elevation of AMPK activation in the fasted state.

To investigate these hypotheses, the following objectives were defined:

1. To determine the effects of CLA isomers, alone or in combination, on hepatic lipid concentration, serum ALT concentration and serum haptoglobin concentration in lean and *fa/fa* Zucker rats.

2. To determine if dietary CLA isomers, alone or in combination, reduce hepatic lipid concentration by reducing levels of active SREBP-1 (SREBP-1 p68), and/or elevating levels of phosphorylated AMPKα (pAMPKα) in livers of fasted lean and *fa/fa* Zucker rats.

Study 2:

It was hypothesized that:

1. t10,c12 CLA reduces hepatic lipid concentration by a mechanism involving suppression of SREBP-1 activation and/or elevation of AMPKα activation in the fed state.

To investigate this hypothesis, the following objective was defined:

1. To measure hepatic protein levels of SREBP-1 p125, SREBP-1 p68, AMPKα and pAMPKα in livers of *fa/fa* Zucker rats after feeding with a meal containing CLA isomers.

MATERIALS AND METHODS

STUDY 1 – FASTED STATE

Animals and Diet

Seventy male *fa/fa* and 70 lean Zucker rats were purchased from Harlan Teklad (Indianapolis, IN) and received at approximately 5 weeks of age. Upon arrival, ten rats (n=10 lean and n=10 *fa/fa* per group) were randomly assigned to one of seven dietary treatments (Table 1). After a 5-8 day acclimatization period, rats began consuming their assigned treatment diets. For the duration of the study, rats were housed in individual wire-bottom cages and exposed to a 14-hour light, 10-hour dark cycle. Humidity was maintained at 55%, and temperature kept between 21 and 23°C. Water was provided *ad libitum*.

Ten lean and 10 *fa/fa* Zucker rats were also obtained at 5 weeks of age, housed under the same conditions, and fed control diet for 1 week. Tissues from these rats were collected at 6 weeks of age and were included to compare baseline values of certain parameters to endpoint values in 14-week-old lean and *fa/fa* Zucker rats fed control diet.

Diet formulations were based on the AIN-93 diet formulated for growth (Table 3) (Reeves, Nelson, & Fahey, 1993). Total fat content of all diets was 8.5% (wt/wt). The sole fat source of the control diet was soybean oil, while the other treatment diets contained soybean oil plus various CLA isomers, either

alone or in combination (Table 4). CLA diets were formulated to contain 0.4% (wt/wt) of c9,t11 CLA and/or t10,c12 CLA (Tables 5-7). CLA used in the diets was in the FFA form.

In addition to the CLA and control treatment groups, pair-weighed groups of lean and *fa/fa* rats were include as a means to determine if changes in measured parameters were due to changes in body weight. As a result, rats in the pair-weighed groups were fed amounts sufficient to maintain body weights that were equal to the lightest CLA-fed lean and *fa/fa* groups.

Diets were prepared bi-weekly in 6 kg batches. Oils were added after the dry ingredients had been pre-mixed to ensure even distribution. After mixing, diets were stored at -20˚C.

Feed intake (adjusted for spillage) was recorded three times per week, and animals received fresh feed at each recording time. If lean and *fa/fa* pair-weighed rats weighed significantly more than the lightest lean or *fa/fa* rat, feed restriction was imposed and pair-weighed rats were fed daily. Body weights were recorded weekly.

Table 2 – Description of Dietary Treatment Groups for Study 1

Dietary Treatments[1]	Description of Lipid Content	Amount (wt/wt)
c9,t11 CLA (9-11)[3]	Purified c9,t11 isomer plus soybean oil	0.4% c9,t11 8.1% soybean oil
t10,c12 CLA (10-12)[4]	Purified t10, t12 isomer plus soybean oil	0.4% t10,c12 8.1% soybean oil
Together (TOG)[3, 4]	Purified c9,t11 **and** purified t10,c12 isomers plus soybean oil	0.4% c9,t11 0.4% t10, c12 7.7% soybean oil
Bioriginals (BIO)[5]	A commercial mixture containing mainly 2 isomers	1.2% CLA mixture providing 0.4% c9,t11 and 0.4% t10,c12 7.3% soybean oil
NuCheck (NCK)[6]	A commercial mixture of mainly 4 CLA isomers	2.2% CLA mixture providing 0.4% c9,t11 and 0.4% t10,c12 6.3% soybean oil
Control (CTL)[7]	Soybean oil only	8.5% soybean oil
Pair-weighed[2, 7] (PW)	Soybean oil only	8.5% soybean oil

[1] n=10 lean and n=10 *fa/fa* rats per group.
[2] Fed the control diet in restricted amounts to maintain body weight equal to the CLA-group with the lowest body weight. This was done to account for the possibility that changes in measured parameters might result from differences in body weight.
[3] c9,t11 oil from Natural ASA, Hovdebygda, Norway.
[4] t10,c12 oil from Natural ASA, Hovdebygda, Norway.
[5] Bioriginals oil from Bioriginals, Saskatoon, Saskatchewan.
[6] NuCheck oil form Nucheck-Prep, Elysian, Minnesota.
[7] Soybean oil from Harlan Teklad, Madison, Wisconsin.

Table 3 – Diet Formulations

Ingredient (g/kg)	9-11	10-12	TOG	BIO	NCK	CTL
Cornstarch[1]	363	363	363	363	363	363
Maltodextrin[2]	132	132	132	132	132	132
Sucrose[2]	100	100	100	100	100	100
Egg white[2]	212.5	212.5	212.5	212.5	212.5	212.5
Cellulose[2]	50	50	50	50	50	50
AIN-93G Mineral Mix[2]	35	35	35	35	35	35
AIN-93G Vitamin Mix[2]	10	10	10	10	10	10
Choline[2]	2.5	2.5	2.5	2.5	2.5	2.5
Biotin mix[2,3]	10	10	10	10	10	10
TBHQ[4]	0.014	0.014	0.014	0.014	0.014	0.014
Lipid[5]	85	85	85	85	85	85

[1] Purchased from Harlan Best Foods, Etobicoke, ON.
[2] Purchased from Harlan Teklad, Madison, WI.
[3] Biotin mix = 200 mg biotin + 1 kg cornstarch; included because avadin in egg white (protein source) binds biotin, therefore extra must be added.
[4] Tert-butyl hydroquinone; purchased from Sigma-Aldrich, St. Louis, MO.
[5] Lipid composition provided in Table 4.

Table 4 – Lipid Composition of Treatment Diets

Dietary Treatment group	Soybean Oil (g/kg diet)	c9,t11 CLA Oil (g/kg diet)	t10,c12 CLA Oil (g/kg diet)	Bioriginals Oil (g/kg diet)	NuCheck Prep Oil (g/kg diet)	TOTAL (g/kg diet)
CTL	85.00	0.00	0.00	0.00	0.00	**85.00**
c9,t11	80.69	4.31	0.00	0.00	0.00	**85.00**
t10,c12	80.67	0.00	4.33	0.00	0.00	**85.00**
TOG	76.36	4.31	4.33	0.00	0.00	**85.00**
BIO	73.00	0.00	0.00	12.00	0.00	**85.00**
NCK	63.00	0.00	0.00	0.00	22.00	**85.00**

Table 5 – Fatty Acid Composition of CLA Oils

	c9,t11 CLA	t10,c12 CLA	Bioriginals	NuCheck Prep
c9,t11[1]	92.7	4.3	32.9	17.8
t10,c12[1]	0.6	92.3	33.2	19.5
t8, c10[1]	0.0	0.0	0.0	18.6
c11, t13[1]	0.0	0.0	0.0	18.7
Other CLA isomers[1,2]	2.7	2.4	7.1	12.7
Total CLA isomers[1]	***96***	***99***	***73.2***	***87.3***
Oleic acid (18:1 c9) [1]	4.0	0.8	12.6	ND[3]
Linoleic acid (c9, c12) [1]	<0.1	<0.1	4.1	ND[3]
Other[1]	0	<0.1	10.1	ND[3]
Total	***100***	***100***	***100***	***100***

[1] Fatty acid composition expressed as a percentage of total fatty acids.
[2] Composition has not been determined.
[3] ND = Not determined.

Table 6 – Percentage of CLA in Oils

	c9,t11 CLA Oil	t10,c12 CLA Oil	Bioriginals Oil	NuCheck Prep Oil
CLA[1]	96.0	99.0	73.2	87.3
Other fatty acids[1]	4.0	1.0	26.8	12.7

[1] CLA content expressed as grams of CLA per 100 grams of oil.

Table 7 – Total Amount of CLA in Each Diet

Dietary Treatment Group	c9,t11 (g/kg)	t10,c12 (g/kg)	t8, c12 (g/kg)	c11, t13 (g/kg)	Other CLA isomers (g/kg)	Total CLA (g/kg)
9-11	4.00	0.03	0.00	0.00	0.12	4.15
10-12	0.19	4.00	0.00	0.00	0.10	4.29
TOG	4.19	4.03	0.00	0.00	0.22	8.44
BIO	3.95	4.00	0.00	0.00	0.85	8.80
NCK	3.91	4.29	4.09	4.11	2.79	19.91

Tissue Collection

Reagents used:

- Phosphate-buffered saline (PBS) stock solution pH 7.4 [8% weight/volume (wt/v) NaCl, 0.02% wt/v KCl, 1.15% wt/v Na_2HPO_4, 0.02% wt/v KH_2PO_4, Fisher Scientific, Whitby, ON; distilled, deionized water (ddH_2O)]
- PBS working solution: 1 part PBS stock solution plus 9 parts ddH_2O

All euthanization procedures used were in accordance with the Canadian Council on Animal Care Guidelines (Olfert et al., 1993). Rats were euthanized by carbon dioxide asphyxiation and cervical dislocation following a 12-hour fast. Final body weights were recorded. Trunk blood was collected after cervical dislocation and was immediately centrifuged at 1500 rpm for 10 minutes at 4°C (Beckman TJ-6R Tabletop Centrifuge, Beckman Instruments, Palo Alto, CA). Serum was aliquotted into 1.5 mL microcentrifuge tubes (cat. # 05-664-3, Fisher Scientific, Whitby, ON) and stored at -80°C. After blood collection, the livers were removed and rinsed in cold PBS working solution. Livers were weighed, wrapped in foil, and snap-frozen in liquid nitrogen before being stored at -80°C.

Hepatic Lipid Concentration

Liver total lipids were extracted by a modified Folch method and quantified gravimetrically (Folch et al., 1957).

Reagents used:

- Chloroform (Optima grade, Fisher Scientific, Whitby, ON)
- Methanol (HPLC grade, Fisher Scientific, Whitby, ON)
- 0.73% wt/v NaCl solution (Fisher Scientific, Fair Lawn, NJ)

One gram of thawed liver was weighed and placed into a 50 mL glass tube containing 22 mL of 2:1 chloroform:methanol. Samples were homogenized for 20 seconds with a Polytron homogenizer (PT MR-2100, 500 watt, Brinkmann Instruments, Rexdale, ON), and the homogenate was filtered through a #1 Whatman filter paper into a 25 mL graduated cylinder. The volume of the eluate was recorded, and 20% of this volume was added as 0.73% NaCl. The graduated cylinder was covered with a stopper, vigorously shaken, and left to settle.

After 24 hours, the chloroform:methanol separated into 2 layers: an upper methanol layer, and a lower chloroform layer containing the lipid. The volume of the chloroform layer was recorded, and the upper methanol layer was removed. Ten mL of the chloroform layer from each sample were placed in a dried and weighed 25 mL glass vial, and the chloroform was evaporated in a heated water

bath (OA-SYS heating system, Organomation Associates, Berlin, MA) with nitrogen gas (0.7 Kg/cm^2) for 1 hour. Vials containing the lipid were cooled overnight in a desiccator and then weighed. The concentration of hepatic lipid was calculated as follows:

$$\text{g lipid/g tissue} = \frac{(\text{dry weight vial + lipid}) - (\text{dry weight vial})}{\text{10 mL chloroform used}} \times \text{volume of chloroform layer}$$

Values were then multiplied by 100 to express the concentration of lipids as a percent.

Radiommunoassays

In radioimmunoassay (RIA), a known concentration of an antigen labelled with a radioactive isotope is incubated with antibody so that the concentration of the antigen binding sites in the system is at a limited and fixed concentration (i.e. only 50% of the total labelled antigen concentration may be bound by antibody). When sample is added, the unlabelled antigen of interest competes with labelled antigen for binding sites. Therefore, the amount of labelled antigen that binds to the antibody decreases as the amount of unlabelled antigen from the sample increases. The bound and unbound antigen fractions are then separated by precipitating the unbound fraction. The radioactivity of the labelled antigen in the bound fraction is counted in a scintillation counter; this radioactivity is inversely proportional to the concentration of unlabelled antigen from the sample. A

standard curve is constructed using known concentrations of labelled antigen and from this curve the amount of antigen in unknown samples can be quantified.

i. Insulin

Five-hour fasting serum was analyzed for insulin with a rat insulin radioimmunoassay kit (Cat. # RI-13K, Linco Research, Inc, St. Charles, MO). All standards, quality controls, samples, non-specific binding (NSB) samples, maximum binding (MB) samples and total count (TC) samples were assayed in duplicate. Serum from lean rats was diluted 1x-2x in assay buffer; serum from *fa/fa* rats was diluted 10x in assay buffer.

Reagents used:

- Assay buffer: 0.05 M PBS pH 7.4 containing 0.025 M EDTA (ethylenediamine tetraacetic acid), 0.08% sodium azide, and 1% RIA grade bovine serum albumin (BSA)
- Rat insulin antibody: guinea pig anti-rat insulin serum in assay buffer
- ^{125}Iodine (^{125}I)-insulin: ^{125}I-insulin label, HPLC purified, hydrated with label hydrating buffer
- Label hydrating buffer: assay buffer containing normal guinea pig IgG as carrier
- Rat insulin standards: 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/mL of purified rat insulin in standard buffer
- Quality controls: purified rat insulin in assay buffer

- Precipitating reagent: goat anti-guinea pig IgG serum, 3% polyethylene glycol (PEG), 0.05 M PBS, 0.025 M EDTA, and 0.08% sodium azide

The assay was carried out over 2 days. On day one, assay buffer was added to 12 x 75 mm borosilicate glass tubes (Fisher Scientific, Whitby, ON) in the following amounts: 200 µL into the NSB tubes and 100 µL into the MB tubes. One-hundred µL of standards, samples, and quality controls were pipetted into the appropriate tubes. One-hundred µL each of ^{125}I-Insulin and rat insulin antibody were then added to MB, standard, sample, and quality control tubes. Tubes were vortexed, covered, and incubated for 20-24 hours at 4°C.

On day two, 1 mL of precipitating reagent was added to all tubes (except the TC tubes), which were then vortexed, incubated for 20 minutes at 4°C, centrifuged for 40 minutes at 2000 x g at 4°C and decanted by inverting the tubes for 30 seconds and blotting excess supernatant. The remaining pellet was counted for radioactivity in a Beckman Gamma 8000 (Scientific Instruments, Irvine, CA) for 1 minute.

The percentage of bound labelled-antigen in each sample was calculated using the following formula:

$$\% \text{ Bound/Total bound} = \frac{(\text{average sample count} - \text{average NSB count})}{(\text{average MB count})} \times 100$$

A standard curve was constructed from the values of the standard tubes using Prism 2.01 Software (Intuitive Software for Science, San Diego, CA).

Using this standard curve, the concentrations of insulin in the samples were determined, and then multiplied by the appropriate dilution factor to obtain the final serum insulin concentrations in ng/mL. Values for each sample expressed in ng/mL were converted to pmol/L using the following formula:

$$\frac{\text{Sample value (ng/mL)}}{5800} \times 1000 \times 1000 = \text{sample value (pmol/L)}$$

ii. C-peptide

Five-hour fasting serum was analyzed for C-peptide with a rat C-peptide radioimmunoassay kit (Cat. # RCP-21K, Linco Research, Inc, St. Charles, MO). All standards, quality controls, samples, NSB samples, MB samples, and TC samples were assayed in duplicate. Serum from lean rats was diluted 10x in assay buffer; serum from *fa/fa* rats was diluted 50x in assay buffer.

Reagents used:

- Assay buffer: 0.05 M PBS pH 7.4 containing 0.025 M EDTA, 0.08% sodium azide, and 1% RIA grade BSA
- Rat C-peptide antibody: guinea pig anti-rabbit C-peptide antibody in assay buffer
- ^{125}I-rat C-peptide: ^{125}I-rat C-peptide label, HPLC purified; hydrated with label hydrating buffer
- Label hydrating buffer: assay buffer containing normal guinea pig serum as carrier

- Rat C-peptide standards: 25, 50, 100, 200, 400, 800, and 1600 pM purified recombinant rat C-peptide in assay buffer
- Quality controls: purified recombinant rat C-peptide in assay buffer
- Precipitating reagent: goat anti-guinea pig IgG serum, 3% PEG, and 0.05% Triton X-100 in 0.05 M PBS, 0.025 M EDTA, 0.08% sodium azide

The assay was carried out over 3 days. On day one, assay buffer was pipetted into 12 x 75 mm borosilicate glass tubes (Fisher Scientific, Whitby, ON) in the following amounts: 300 µL into NSB tubes, 200 µL into MB tubes, and 100 µL into standard, sample and quality control tubes. One-hundred µL of standards, samples, and quality controls were then added to the appropriate tubes. Finally, 100 µL of rat insulin antibody was added to MB, standard, sample and quality control tubes, which were then vortexed, covered, and incubated for 20-24 hours at 4°C.

On day two, 100 µL of ^{125}I-rat C-peptide was added to all tubes, which were then vortexed, covered, and incubated for 22-24 hours at 4°C.

On day three, 1 mL of cold precipitating reagent was added to all tubes (except the TC tubes), which were then vortexed, incubated for 20 minutes at 4°C, centrifuged for 40 minutes at 2000 x g at 4°C and decanted by inverting the tubes for 30 seconds and blotting excess supernatant. The remaining pellet was counted for radioactivity in a Beckman Gamma 8000 (Scientific Instruments, Irvine, CA) for 1 minute.

The percentage of the bound labelled antigen in each sample was calculated using the following formula:

$$\% \text{ Bound/Total bound} = \frac{(\text{average sample count} - \text{average NSB count})}{(\text{average MB count})} \times 100$$

A standard curve was constructed from the values of the standard tubes using Prism 2.01 Software (Intuitive Software for Science, San Diego, CA). Using this standard curve, the concentrations of C-peptide in the samples were determined, and then multiplied by the appropriate dilution factor to obtain the final serum C-peptide concentrations in pmol/L.

iii. Adiponectin

Adiponectin was quantified from termination serum using a mouse adiponectin RIA kit (Cat. #MADP-60HK, Linco Research, Inc., St. Charles, MO). At the time of testing, no commercial rat adiponectin kits were available; however, this mouse kit was chosen for its ability to detect adiponectin in rat as well as mouse serum. All standards, quality controls, samples, NSB samples, MB samples, and TC samples were assayed in duplicate. Serum from lean and *fa/fa* Zucker rats was diluted 500x in assay buffer.

Reagents used:

- 10x assay buffer: diluted with 450 mL ddH_2O; final concentration after dilution is 10 mM phosphate buffer pH 7.6 containing 0.08% sodium azide, 0.1% RIA grade BSA
- Adiponectin antibody: rabbit anti-adiponectin antibody
- ^{125}I-adiponectin: ^{125}I-adiponectin label; rehydrated with 13.5 mL assay buffer
- Murine adiponectin standards: 100 ng/mL; rehydrated with 1 mL ddH_2O; serially diluted to produce additional standards ranging from 0.78-50 ng/mL
- Quality controls: purified recombinant adiponectin; rehydrated with 1 mL ddH_2O
- Rabbit carrier: 30% normal rabbit serum
- Precipitating reagent: goat anti-rabbit IgG serum, 3% PEG and 0.05% Triton X-100 in 0.05M PBS, 0.025M EDTA, 0.08% sodium azide

This assay was carried out over 2 days. On day one, assay buffer was pipetted into 12 x 75 mm borosilicate glass tubes (Fisher Scientific, Whitby ON) in the following amounts: 300 µL into the NSB tubes, 200 µL into the MB tubes, and 100 µL into the standard, sample, and quality control tubes. One-hundred µL of standards, samples, and quality controls were added to the appropriate tubes. One-hundred µL each of ^{125}I-Adiponectin and adiponectin antibody were added to each tube (except TC tubes, which received only ^{125}I-Adiponectin).

Tubes were vortexed, covered, and incubated for 20-24 hours at room temperature.

On day two, 10 µL of rabbit carrier and 1 mL of cold precipitating reagent were added to all tubes (except TC tubes), which were then vortexed, incubated for 20 minutes at 4°C, centrifuged for 40 minutes at 2000 x g, and decanted by inverting tubes for 30 seconds and blotting excess supernatant. The remaining pellet was counted for radioactivity in a Beckman Gamma 8000 (Scientific Instruments, Irvine, CA) for 1 minute.

The percentage of the bound labelled antigen in each sample was calculated using the following formula:

$$\% \text{ Bound /Total bound} = \frac{(\text{sample count} - \text{NSB count})}{\text{MB count}} \times 100$$

A standard curve was constructed from the values of the standard tubes using Prism 2.01 Software (Intuitive Software for Science, San Diego, CA). Using this standard curve, the concentrations of adiponectin in the samples were determined, and then multiplied by the appropriate dilution factor to obtain the final serum adiponectin concentrations in ng/mL.

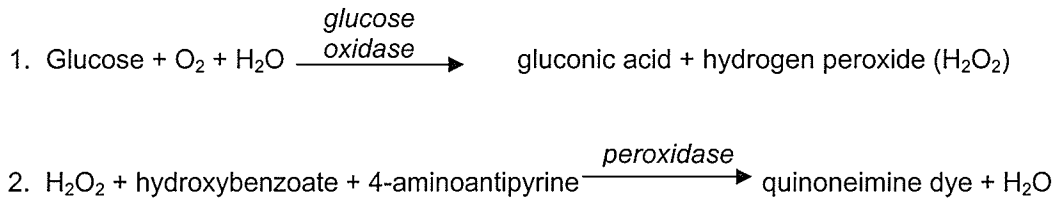
Endpoint Enzyme-Mediated Colourimetric Assays

In endpoint enzyme-mediated colourimetric assays, enzymatic reactions are used to convert the analyte of interest into a coloured product that can be

detected spectrophotometrically. If the product of the initial reaction is not directly detectable, the first enzymatic analyte-specific reaction is coupled to an indicator reaction which quantitatively converts the product of the first reaction to a final product that can be detected. In most cases, this final product is a coloured dye, the absorbance of which, at a specified wavelength, is proportional to the concentration of the analyte in the original sample.

i. Glucose

Five-hour fasting serum was analyzed for glucose using a commercial kit (Cat. # 220-32, Diagnostic Chemicals Ltd., Charlottetown, P.E.I.), the principle of which is modified from the Trinder glucose oxidase/peroxidase method (Trinder, 1969):



The quinoneimine dye produces a colour, the intensity of which is proportional to the concentration of glucose in the sample.

Reagents used:

- Glucose colour reagent: a solution containing a buffer (pH 7.25 at 25°C, 0.25 mmol/L 4-aminoantipyrine, 20 mmol p-hydroxybenzoate, >40 000

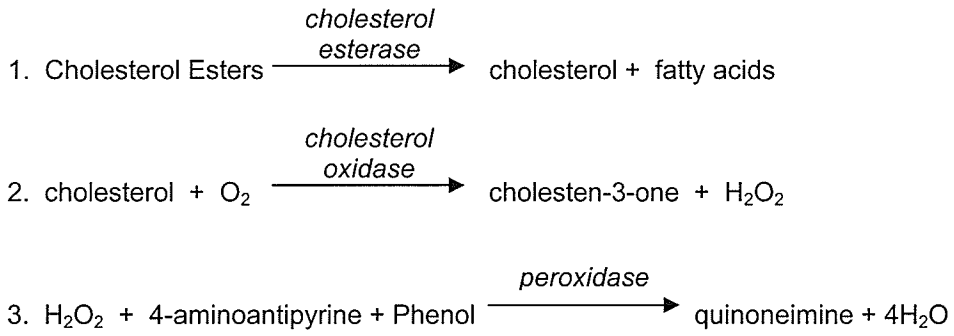
U/L glucose oxidase (Aspergillus niger), >2000 U/L horseradish peroxidase, and preservatives. This was reconstituted with ddH_2O.

- Glucose Standard: a solution containing 5 mM glucose and preservatives; serially diluted to produce 3 additional standards (0.625, 1.25, and 2.5 mM)

Serum from lean and *fa/fa* rats was diluted 5x with deionized water. Five µL of standard, sample, blank (ddH_2O), and quality control were pipetted, in triplicate, into the wells of a microplate (Costar EIA/RIA 96-well polystyrene plate, Fisher Scientific, Whitby, ON). Two-hundred µL of reconstituted glucose colour reagent was then added to each well. After gentle shaking, the plate was incubated at room temperature for 10 minutes, after which the absorbance of the colour in each well at 505 nm was read in a microplate reader (SpectraMax 340, Molecular Devices Corp., Sunnyvale, CA) using SOFTmax Pro software (version 1.2.0, Molecular Devices Corp., Sunnyvale, CA). The mean result for sample was multiplied by the dilution factor to obtain the final serum glucose concentration in mmol/L. The standard curve of all assays had a correlation coefficient of 0.9 or greater, and samples with coefficients of variation greater than 10% were re-assayed.

ii. Cholesterol

Serum cholesterol was quantified in termination serum using a commercial kit (Cat. # 225-26, Diagnostic Chemicals Ltd., Charlottetown, P.E.I), the principle of which is adapted from Allain et al. (1974) and Roschlau et al. (1974):



The quinoneimine dye produces a colour, the intensity of which is directly proportional to the concentration of total cholesterol in the sample.

Reagents used:

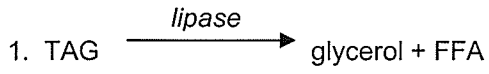
- Cholesterol colour reagent: a solution containing phosphate buffer (pH 6.7 at 25°C), 1.6 mM 4-aminoantipyrine, >5560 U/L horseradish peroxidase, >400 U/L cholesterol oxidase (Nocardia sp.), a preservative, and a stabilizer
- Cholesterol phenol reagent: a solution containing 40 mM phenol, a surfactant, and a stabilizer
- Cholesterol working reagent: equal volumes of cholesterol colour reagent and cholesterol phenol reagent, mixed gently and thoroughly

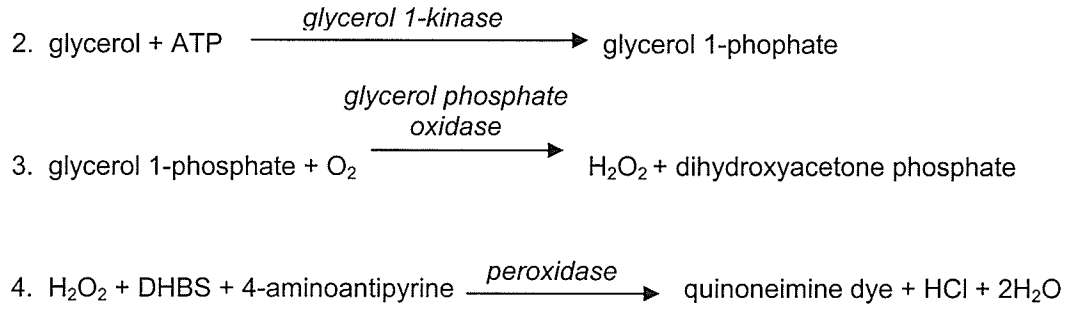
- Cholesterol Standard: a solution containing 5 mM cholesterol, a surfactant, and a preservative; serially diluted to produce 3 additional standards (0.625, 1.25, and 2.5 mM)

Serum was diluted 5x for lean rats and 9x for *fa/fa* rats using PBS working solution. Ten µL of standard, sample, blank (ddH_2O), and Quality Control were pipetted, in triplicate, into the wells of a microplate (Costar EIA/RIA 96-well polystyrene plate, Fisher Scientific, Whitby, ON). Two-hundred µL of working reagent was added to each well, and the plate was mixed gently for 30 seconds before incubation at 37°C. After 5 minutes, the absorbance of the colour in each well was read at 505 nm with a microplate reader (SpectraMax 340, Molecular Devices Corp., Sunnyvale, CA) using SOFTmax Pro software (version 1.2.0, Molecular Devices Corp., Sunnyvale, CA). The mean result for each sample was multiplied by the appropriate dilution factor to obtain the final serum cholesterol concentration in mmol/L. The standard curve of all assays had a correlation coefficient of 0.9 or greater, and samples with coefficients of variation greater than 10% were re-assayed.

iii. TAG

Serum TAG was quantified from termination serum using a commercial kit (Cat. # 210-75, Diagnostic Chemicals Ltd., Charlottetown, P.E.I.), the principle of which is based on Fossati and Lorenzo (1982) and McGowan et al. (1983):





The quinoneimine dye produces a colour, the intensity of which is directly proportional to the concentration of TAG in the sample.

Reagents used:

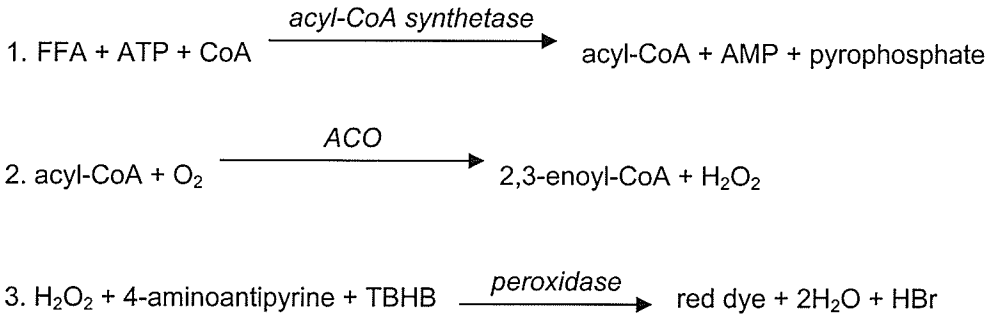
- Triacylgycerol colour reagent: a solution (after reconstitution with ddH_2O) containing 3.0 mM ATP, 0.66 mM DHBS, 0.7 mM 4-aminoantipyrine, 2400 U/L peroxidase, 5000 U/L glycerol phosphate oxidase, 300 U/L glycerol kinase, 250 000 U/L lipase, and a stabilizer
- Triacylglycerol buffer: a 100 mM Pipes buffer (pH 7.6 at 25°C) containing 2.0 mM magnesium chloride, a surfactant, and a preservative
- Triacylglycerol standard: a solution containing glycerol equivalent to 2.0 mM triolein, a surfactant, and a preservative; serially diluted to produce 3 additional standards (0.25, 0.5, and 1 mM)

Serum was diluted 10x for *fa/fa* rats and 4x for lean rats using 0.9% saline. Ten µL of standards, samples, and blank (0.9% saline) were pipetted, in triplicate,

into the wells of a microplate (Costar EIA/RIA 96-well polystyrene plate, Fisher Scientific, Whitby, ON). TAG colour reagent (100 µL) and ddH_2O (140 µL) were added to each well; the plate was then mixed gently and incubated at room temperature. After 15 minutes, the absorbance of the colour in each well was read at 515 nm with a microplate reader (SpectraMax 340, Molecular Devices Corp., Sunnyvale, CA) using SOFTmax Pro software (version 1.2.0, Molecular Devices Corp., Sunnyvale, CA). The mean result for each sample was multiplied by the appropriate dilution factor to obtain the final serum TAG concentration in mmol/L. The standard curve of all assays had a correlation coefficient of 0.9 or greater, and samples with coefficients of variation greater than 10% were re-assayed.

iv. FFA

Termination serum was analyzed for fasting serum FFA using a commercial kit (Cat. #11 383 175 001, Roche Diagnostics, Penzberg, Germany). The protocol had previously been adapted for use in a microplate reader (Gillam, 2003). The principle of the assay is as follows:



Reagents used:

- Reaction mixture A: 1 tablet A (containing ATP, CoA, acyl-CoA synthetase, peroxidase, ascorbate oxidase, 4-aminoantipyrine and stabilizers) dissolved in 11 mL potassium phosphate buffer pH 7.8
- Reaction mixture B: 1 tablet B (containing ACO and stabilizers) dissolved in 0.6 mL ACO dilution solution
- Solution C: aqueous N-ethyl-maleinimide solution with stabilizers

Two-hundred µL of reaction mixture A was pipetted into each well of a Costar EIA/RIA 96-well polystyrene plate (Fisher, Whitby, ON). Ten µL each of ddH_2O (blank), quality control and serum samples were placed, in triplicate, into each well. The plate was then placed into a microplate reader (SpectraMax 340, Molecular Devices Corporation, Sunnyvale, CA), mixed for 30 seconds and incubated at 22°C for 10 minutes.

Ten µL of solution C was added to each well, the plate was mixed for 30 seconds in the microplate reader and the absorbance was read at 546 nm using SOFTmax Pro software (version 1.2.0, Molecular Devices Corp., Sunnyvale, CA). This absorbance was designated A_1. After the absorbance was read, 10 µL of reaction mixture B was added to each well, the plate was mixed in the microplate reader for 30 seconds, and incubated at 22°C for 20 minutes. The plate was again mixed for 30 seconds, and the absorbance was read at 546 nm using SOFTmax Pro software (version 1.2.0, Molecular Devices Corp., Sunnyvale, CA). This absorbance was designated A_2.

Serum FFA concentrations were calculated using the following formula:

$$C\ (mmol/L) = \frac{V}{\varepsilon \times d \times v} \times \Delta A$$

Where:

C = concentration of FFA in the sample

V = final sample volume in mL (0.230 mL)

v = sample volume in mL (0.010 mL)

d = light path in cm (0.53326 cm)

ε = absorption coefficient at 546 nm [19.3 x (1 x $mmol^{-1}$ x cm^{-1})^3]

ΔA = change in absorbance of the sample minus the change in absorbance of the blank, $(A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}$

v. Haptoglobin

Termination serum was analyzed for fasting serum haptoglobin concentrations using a commercial kit (Cat. #TP 801, Phase™ Range Haptoglobin Assay, Tri-Delta Diagnostics, Inc., Ireland). This assay employs the intrinsic peroxidase activity of haemoglobin to produce a coloured dye that can be read spectrophotometrically. Haptoglobin present in the sample binds to haemoglobin and preserves its peroxidase activity. When hydrogen peroxide (H_2O_2) and a chromogen are added, the peroxidase from haemoglobin reacts with H_2O_2 to produce a coloured dye.

$$\text{haptoglobin} + \text{haemoglobin} + H_2O_2 + \text{chromogen} \xrightarrow{\textit{peroxidase}} \text{coloured dye}$$

The absorbance of the dye at 603 nm (which represents the preservation of the peroxidase activity of haemoglobin) is directly proportional to the amount of haptoglobin in the sample.

Reagents used:

- Reagent 1: equal parts stabilized haemoglobin and haemoglobin diluent
- Reagent 2: a 9:5 mixture of chromogen reagent and substrate containing stabilized H_2O_2
- Haptoglobin standard: 2 mg/mL haptoglobin; serially diluted with sample/standard diluent to produce 3 additional standards (0.25, 0.5, and 1.0 mg/mL)
- Sample/standard diluent: PBS working solution

Serum from *fa/fa* Zucker rats was diluted 2x with PBS working solution to allow for accurate quantification from the standard curve. Samples and standards (7.5 µL) were pipetted, in duplicate, into wells of a Costar EIA/RIA 96-well polystyrene plate (Fisher, Whitby, ON). One-hundred µL of reagent 1 was added to each well and the plate was gently mixed. Then 140 µL of reagent 2 was added to each well, and the plate was incubated at room temperature for 5 minutes. The absorbance at 630 nm was then read with a microplate reader (SpectraMax 340, Molecular Devices Corp., Sunnyvale, CA) using SOFTmax Pro software (version 1.2.0, Molecular Devices Corp., Sunnyvale, CA). The mean

result for each rat was multiplied by the dilution factor to obtain the final serum haptoglobin concentration in mmol/L. The standard curve of all assays had a correlation coefficient of 0.9 or greater, and samples with coefficients of variation greater than 10% were re-assayed.

Kinetic Enzyme-Mediated Colourimetric Assays

Kinetic enzyme-mediated colourimetric assays also employ enzymes to drive reactions that produce coloured products, however, instead of the final absorbance of the coloured-product being measured, the change in absorbance from one time point to another (i.e. the rate of the reaction) is used to determine the concentration of the analyte in the sample. The rate of the reaction is proportional to the concentration of the analyte in the sample.

i. ALT

Concentrations of serum ALT were quantified using a spectrophotometric assay (Diagnostic Chemicals Ltd., Charlottetown, P.E.I.). The principle of the assay is as follows:

1. alpha-ketoglutarate + L-alanine $\xrightarrow{ALT}$ L-glutamate + Pyruvate

2. Pyruvate + NADH + H^+ $\xrightarrow{\text{Lactate dehydrogenase}}$ Lactate + NAD^+

Reagents used:

- ALT-SL enzyme reagent: a solution containing a buffer (pH 7.5 at 25°C), 500 mM L-alanine, >1200 U/L lactate dehydrogenase (bacterial), and a preservative
- ALT-SL substrate reagent: a solution containing 15 mM 2-oxoglutarate, 0.18 mM NADH, and a preservative
- Working reagent: 4 parts ALT-SL enzyme reagent plus 1 part ALT-SR substrate reagent

The spectrophotometer was blanked with ddH_2O. Fifty µL of serum was pipetted into a disposable cuvette. One µL of working reagent was added to each cuvette, mixed, and incubated at 37° for 3-5 minutes. When the change in absorbance was constant, the absorbance at 340 nm was recorded at one minute intervals.

Serum ALT concentrations were determined using the following formula:

$$\text{ALT (U/L)} = \frac{\Delta A/\text{min} \times \text{assay volume (mL)} \times 1000}{6.22 \times \text{light path (cm)} \times \text{sample volume (mL)}}$$

Where: ΔA/min = change in absorbance per minute

Assay volume = total reaction volume expressed in mL

1000 = converts U/mL to U/L

6.22 = absorbance coefficient of NADH at 340 nm

Light path = length of the light path expressed in cm

Sample volume = sample volume expressed in mL

Liver Protein Extraction and Quantification

i. Extraction

Reagents used:

- 3x sample buffer: 0.2 M Tris-HCl (Roche, Indianapolis, IN), 3% SDS Sigma-Aldrich, St. Louis, MO), 30% glycerol (Fisher Scientific, Fair Lawn, NJ), ddH_2O

To extract total protein from liver samples, 30 mg of liver was powdered under liquid nitrogen with a mortar and pestle. Thirty µL of 3x sample buffer per mg of liver was added to and mixed with the sample, which was then left at room temperature for 15 minutes to allow for complete lysis of hepatocytes. Samples were then transferred to 1.5 mL microcentrifuge tubes (cat. # 05-664-3, Fisher Scientific, Fair Lawn, NJ) and centrifuged for 20 minutes at 13 000 rpm (Biofuge

13, Heraeus Instruments). The supernatant containing the protein was removed, sonicated for 10 seconds, and stored at -80°C until analyzed.

ii. Quantification

Protein concentration of liver samples was determined using the Pierce bicinchoninic acid (BCA) assay (Cat. #23225, Pierce, Rockford, IL). This assay is based on two reactions: (1) Reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline environment (known as the biuret reaction), and (2) Formation of a purple complex when Cu^{1+} combines with BCA.

1. Protein (peptide bonds) + $Cu^{2+} \xrightarrow{OH^-}$ tetradenate-Cu^{1+} complex
2. Cu^{1+} + BCA → BCA-Cu^{1+} ternary complex (purple colour)

The intensity of the purple colour produced, when measured at 550 nm, is directly proportional to the concentration of protein in the original sample.

Reagents used in the assay:

- Reagent A: sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.2 M sodium hydroxide
- Reagent B: 4% cupric sulphate
- Protein standard: 2 mg/mL BSA (Cat. # 23209, Pierce, Rockford, IL); diluted with ddH_2O to produce 5 additional standards (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL)

Liver extracts were diluted 4x with ddH_2O. Briefly, 10µL of standards and sample were pipetted, in triplicate, into the wells of a 96-well plate (Nunc Nalge International, Roskitde, Denmark). Depending on the number of samples to be assayed, appropriate volumes of Reagent A and Reagent B were mixed to achieve a ratio of approximately 221 µL of Reagent A to 4 µL of Reagent B for each well. Two-hundred µL of the mixture of Reagents A and B was added to each well. After 30 minutes of incubation at 37°C, the absorbance of the purple colour in each well at 550 nm was read on a microplate reader (Thermomax, Molecular Devices Corp., Sunnyvale, CA) using SOFTmax Pro software (version 2.34, Molecular Devices, Corp., Sunnyvale, CA). The final protein concentration of liver samples was determined by multiplying the results for each sample by the dilution factor.

Identification of Hepatic Proteins

i. Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Once hepatic proteins were extracted, SDS-PAGE was used to separate the proteins based on molecular mass. By using polyacrylamide gels containing SDS, proteins are denatured and thus can be separated based on molecular mass alone. Exposing the gel to an electric field (electrophoresis) draws the proteins through the porous gel matrix, with proteins of higher molecular mass migrating more slowly through the gel than those with lower molecular mass.

Reagents used:

7.5% separating gel:

- 7.5% (wt/v) acrylamide (Sigma-Aldrich, St. Louis, MO)
- 0.1% (wt/v) SDS (Sigma-Aldrich, St. Louis, MO)
- 0.34 M Tris•HCl pH 8.8 (Roche, Indianapolis, IN)
- 0.1% (wt/v) ammonium persulfate (BioRad, Richmond, CA)
- 8×10^{-4}% N, N, N', N'-Tetramethylethylenediamine (TEMED)
- ddH_2O

5% stacking gel:

- 5% (wt/v) acrylamide (Sigma-Aldrich, St. Louis, MO)
- 0.1% (wt/v) SDS (Sigma-Aldrich, St. Louis, MO)
- 0.13 M Tris•HCl pH 6.8 (Roche, Indianapolis, IN)
- 0.1% (wt/v) ammonium persulfate (BioRad, Richmond, CA)
- 0.25% TEMED
- ddH2O

H_2O-saturated butanol

0.5% (wt/v) bromophenol blue

0.5% (v/v) β-mercaptoethanol

SDS-PAGE electrode buffer: 0.125 M Tris (Roche, Indianapolis, IN), 0.96 M glycine (Fisher Scientific, Fair Lawn, NJ), 0.5% SDS (Sigma-Aldrich, St. Louis, MO)

One mm spacers were set up between a large and small glass plate. The glass plates were then inserted into a sandwich clamp assembly and placed into a casting stand. Components for the 7.5% separating gel were mixed and poured between the two glass plates. A small amount of H_2O-saturated butanol was gently pipetted over the top of the separating gel to ensure the top of the gel was even. After the stacking gel had completely polymerized (approximately 1 hour), the butanol was poured off and the gel was rinsed with ddH_2O. A 10-well comb was placed between the glass plates. Components for the 5% separating gel were mixed and poured on top of the separating gel, between the 10-well comb. The separating gel was left to polymerize for 30 minutes.

After polymerization of the stacking gel, the sandwich clamp assembly containing the gel was transferred to an electrophoretic apparatus, which was placed in a transparent buffer tank. A portion of the SDS-PAGE electrode buffer was poured into the middle of the electrophoretic apparatus, and the rest was poured around the electrophoretic apparatus into the buffer tank.

Using the concentrations from the protein assay, the volume of protein sample required to load 20 µg of protein per well was calculated. Samples were mixed with 0.5 µL of bromophenol blue and 0.5 µL of β-mercaptoethanol and placed in a 190°F pre-heated water bath for 8 minutes to denature the proteins. Samples and molecular mass markers (Benchmark Pre-Stained Protein Ladder, BioRad, Richmond, CA) were then loaded onto the gels using a glass syringe. To account for variation in intensity of film development, an additional sample isolated from the same lean PW animal was loaded onto every gel. The

electrophoretic apparatus was then connected to a power supply (Power Pak, BioRad, Richmond, CA), and electrophoresis was conducted at 20 mA per gel for approximately 90 minutes.

ii. Gel Transfer

After proteins were separated, they were electrophoretically transferred to 6.8 cm x 8 cm polyvinylidene difluoride (PVDF) membranes (Roche, Indianapolis, IN).

Reagents used:

- Transfer buffer: 20% methanol (optima grade, Fisher Scientific, Whitby, ON), 25 mM Tris (Roche, Indianapolis, IN), 130 mM glycine (Fisher Scientific, Whitby, ON), ddH_2O
- 5x Tris-buffered saline in Tween-20 (TBST): 0.1 M Tris-HCl pH 7.4 (Roche, Indianapolis, IN), 0.75 M NaCl (Sigma-Aldrich, St. Louis, MO), 0.25% Tween-20 (Sigma-Aldrich, St. Louis, MO)
- 1x TBST: 1 part 5x TBST plus 4 parts ddH_2O

After SDS-PAGE was complete, the sandwich clamp assembly was dismantled and one of the glass plates was removed from the gel. The stacking gel was discarded. To remove the gel from the second glass plate, a piece of blotting paper was allowed to adhere to the gel, and used to gently peel the gel off the glass. A PVDF membrane, equilibrated in transfer buffer for 5 minutes,

was then placed on top of the gel. The membrane was covered with another piece of blotting paper. Fiber pads were placed on either side of the blotting paper/membrane "sandwich", which was then placed inside a transfer cassette, with the membrane closer to the positive side. The cassette was closed and placed into an electrode module with the negative side facing the appropriate negative side of the module. Transfer buffer was poured into the buffer tank. To prevent the gel from melting, a stir bar and ice pack were placed in the buffer tank. Protein transfer was achieved by electrophoresis at 100 V for 1 hour, with cooling and stirring. Membranes containing the transferred proteins were placed in a container with 1x TBST and stored at 4°C.

iii. Western Immunoblotting

Western immunoblotting uses antibodies to detect a protein of interest in a mixture of proteins after separation by gel electrophoresis. Briefly, the membrane is incubated with a primary antibody, which binds to the protein of interest. After a washing step, the membrane is incubated with a secondary antibody conjugated to an enzyme, which attaches itself to the primary antibody. Alkaline phosphatase or horseradish peroxidase is conjugated to the secondary antibody, and when exposed to a chemiluminescent agent, this enzyme catalyzes the production of a luminescent compound detectable upon exposure to autoradiographic film.

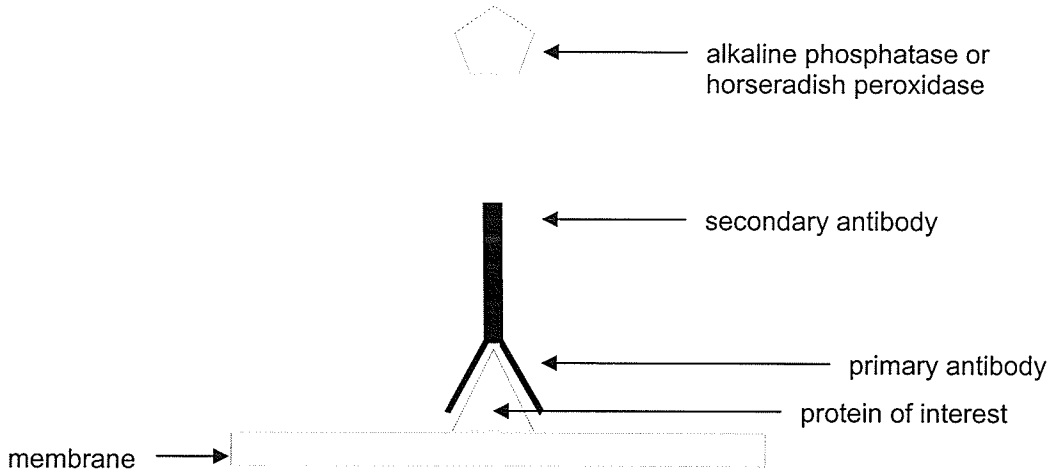


Figure 3. Visual representation Western immunoblotting.

Reagents used:

- 3% (wt/v) BSA in 5x TBST
- 1% (wt/v) BSA in 5x TBST
- 1x TBST
- ECL Plus Western Blotting Detection System (Cat. # RPN2132, Amersham Biosciences, Buckinghamshire, UK)

All blocking and incubation steps were carried out on an orbital shaker. To prevent antibody binding to non-specific binding sites, PVDF membranes were placed in 10 mL of 3% BSA-TBST for 1 hour and 15 minutes. The 3% BSA-TBST was removed, and a primary antibody to the protein of interest, in 3% BSA-TBST, was added (Table 8). The membranes were incubated with the primary antibody for 1 hour. Membranes were then rinsed and washed with 1x TBST 4 times for 5 minutes each (total washing time of 20 minutes). The

membrane was then incubated with secondary antibody in 1% BSA-TBST (Table 8). Following this incubation period, membranes were again rinsed and washed for 20 minutes. Membranes were then immersed in chemiluminescent reagent (ECL Plus Western Blotting Detection System), placed between acetate sheets and exposed to blue X-ray film (Kodak Scientific Imaging Film, Cat. # 864 6770, Kodak, Rochester, NY). Film was developed with developer and fixer (Kodak, Rochester, NY). Relative intensity of the protein bands was quantified with a GS-800 Calibrated Densitometer and Quantity One software (Bio-Rad Laboratories, 1-D Analysis Service, Mississauga, ON).

To account for variation in sample loading, each membrane was stripped (see below for stripping procedure) and blotted with eEF_2. Levels of eEF_2 have been used previously in SDS-PAGE analysis to correct for variability in sample loading (Wang et al., 2001; Smith et al., 2005). For all hepatic proteins measured, results are expressed relative to levels of the intensity control (lean PW 119 sample from the corresponding gel) and loading control (hepatic eEF_2) using covariate statistics.

iv. Stripping of Membrane Blots

Membranes were stripped after western immunoblotting to allow the membranes to be probed with other antibodies.

Reagents used:

- Stripping buffer: 20% SDS; 0.5M Tris•HCl pH 6.8

- 0.5% (wt/v) β-mercaptoethanol
- 1x TBST
- 10% bleach

Two membranes were placed together in a small, clean plastic container. Twenty-five mL of stripping buffer was added to the container. In the fume hood, 80 µL of β-mercaptoethanol per 10 mL of stripping buffer was added to the container. The container was then closed and placed into a larger container, which was then sealed and placed on an orbital shaker overnight. The next day, the stripping buffer solution was poured down the fume hood sink, and the membranes were placed into a new clean container. They were then rinsed with 1x TBST, and agitated for 5 minutes. This washing procedure was repeated until no odour could be detected.

Table 8 – Antibodies used in Western Immunoblotting Analysis

Primary Antibody	Name	Source	Dilution[1]	Host	Secondary Antibody	Dilution[2]	Molecular Weight (kDa)
AMPK	AMP-activated protein kinase	Cell Signaling, Cat. # 2532	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	62
pAMPKα	Phospho-AMPK-α (Thr172)	Cell Signaling, Cat. # 2531	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	62
SREBP-1	Sterol regulatory element-binding protein 1 (C-20)	Santa Cruz Biotechnology, Inc., Cat. # sc-366	1:500	Rabbit	Anti-rabbit horseradish peroxidase[4]	1:10 000	125 (inactive) 68 (active)
eEF2 (loading control)	Eukaryotic elongation factor 2	Cell Signaling, Cat. # 2332	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	100

[1] Primary antibodies diluted with 3% BSA-TBST
[2] Secondary antibodies diluted with 1% BSA-TBST
[3] Membranes incubated in secondary antibody for 1 hour
[4] Membranes incubated in secondary antibody for 45 minutes

Statistical Analysis

Statistical analysis was performed using SAS software version 9.1 (SAS Institute Inc., Cary, NC). For comparison of baseline and endpoint values, two-way analysis of variance (ANOVA) was used to detect differences in genotype, time, and genotype x time interactions. If significant differences in genotype, time, or genotype x time interactions were detected, pre-planned contrast statements were used to examine differences among specific groups.

For comparison of CLA treatments in lean and *fa/fa* Zucker rats, two-way ANOVA was used to examine the differences among genotype, diet, and genotype x diet interactions. If significant genotype x diet interactions were detected, pre-planned contrast statements were used to carry out specific comparisons among groups. Liver weight adjusted for body weight (adjusted liver weight) was analyzed using two-way analysis of covariance (ANCOVA), with body weight as the covariate. Results from Western Immunoblotting were analyzed using ANCOVA, with values for the intensity and loading controls used as covariates. In the case of SREBP-1 and AMPK activation, the sum of the active and non-active protein was used as an additional covariate. Correlation between parameters was calculated using Pearson's Correlation.

For analyses of parameters in which sample size of each dietary group was unequal, normality and homogeneity of variance was tested using the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance. For parameters in which sample size for each dietary group was equal, ANOVA

was considered robust and reasonable deviations from normality and homogeneity of variance were disregarded (Hassard, 1991; Roberts, 1999).

Differences having a p-value of 0.05 or less were considered significant. In the case of contrast statements, 0.05 was divided by the number of contrast statements to determine the appropriate level of significance for that number of comparisons.

STUDY 2 – FED STATE

The objective of Study 2 was to assess possible differences in hepatic AMPK and SREBP-1 activation in the fed state relative to the fasted state.

Animals and Diet

Twenty-eight male *fa/fa* and 7 lean Zucker rats were purchased from Harlan Teklad (Indianapolis, IN) and received at approximately 5 weeks of age. Rats were randomly assigned to one of five treatment groups: fa 9-11 fed, fa 10-12 fed, fa CTL fed, fa CTL fasted or ln CTL fasted. Each group consisted of seven rats. Rats began consuming treatment diets after a 5-7 day acclimatization period. Rats consumed treatment diets for 8 weeks.

Rats were housed in individual wire-bottom cages and exposed to a 14-hour light, 10-hour dark cycle. Humidity was maintained at 55%, and temperatures kept between 21 and 23°C. Water was provided *ad libitum*.

Diet formulations for the fa 9-11 fed, fa 10-12 fed, CTL groups were identical to the c9,t11, t10,c12, and CTL diets used in Study 1 (see Tables 2-7). Feed intake (adjusted for spillage) was recorded three times per week, and animals received fresh feed at each recording time. Body weights were recorded weekly.

Tissue Collection and Analyses

Rats in the fa CTL fasted group were euthanized by carbon dioxide asphyxiation following a 12-hour fast. The fa 9-11 fed, fa 10-12 fed, fa CTL fed, and ln CTL fed groups, which were to be terminated in the fed state, were euthanized following a 2-hour re-feed period. A 12-hour fast followed by a 2-hour re-feeding period was chosen based on results from preliminary studies (see Appendices 3, 4, and 5). Feed dishes for these groups were removed before their scheduled termination time and weighed. After a 12-hour fast, dishes were returned 2 hours prior to their scheduled termination time. Re-fed rats were euthanized immediately following the re-feeding period. Feed dishes were weighed after rats were terminated to measure the amount of food consumed during the re-feed period. Final body weights, blood, and livers were collected, processed, and stored as in Study 1.

Hepatic lipid content, liver protein extraction, SDS-PAGE, gel transfer and Western immunoblotting procedures were carried out as in Study 1.

Statistical Analysis

Statistical analysis was performed using SAS software version 9.1 (SAS Institute Inc., Cary, NC). One-way ANOVA was used to examine differences among the dietary treatment groups. If ANOVA showed a significant effect of

group, pre-planned contrast statements were used to carry out specific comparisons among groups. Liver weight adjusted for body weight was analyzed using ANCOVA, with body weight as the covariate. Results from Western Immunoblotting were analyzed using one-way ANCOVA, with values for intensity and loading controls used as covariates. In the case of SREBP-1 and AMPK activation, the sum of active and non-active protein was used as an additional covariate.

In analyses of parameters in which sample size of each dietary group was unequal, normality and homogeneity of variance was tested using the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance. If data were not normally distributed or did not have homogenous variance, data was log transformed. For parameters in which sample size for each dietary group was equal, ANOVA was considered robust and reasonable deviations from normality and homogeneity of variance were disregarded (Hassard, 1991; Roberts, 1999).

Differences having a p-value of 0.05 or less were considered significant. In the case of contrast statements, 0.05 was divided by the number of contrast statements to determine the appropriate level of significance for that number of comparisons.

SUMMARY OF PREVIOUS RESEARCH

Study 1 of the current project was part of a larger research study conducted in our laboratory in 2003. As such, other researchers from our laboratory previously have published results regarding the effects of dietary CLA isomers on some of the metabolic abnormalities associated with MetS. These results, although previously reported, are relevant to this dissertation to provide a complete picture of how dietary CLA isomers affect MetS. Therefore, the following tables present the results from previous analysis for fasting serum leptin, adiponectin, insulin, C-peptide, TAG, FFA, and oral glucose tolerance [assessed by area under the curve for glucose (AUCg) after oral glucose tolerance testing (OGTT)]. Details of procedures and statistical analyses for fasting serum leptin and adiponectin are provided in Zirk (2005), while those for fasting serum insulin, C-peptide, TAG, FFA and oral glucose tolerance can be found in Diakiw (2005).

Table 9 – Fasting Serum Adiponectin Concentrations (Based on Genotype) of Lean and *fa/fa* Zucker Rats fed CLA-containing or Control Diets for 8 Weeks (Adapted from Zirk, 2005).

	Lean	*fa/fa*
Serum Adiponectin (ng/mL)	4471 ± 139	6025 ± 179^

^ Denotes a significant genotype difference ($p<0.0001$).
Data are expressed as mean ± SEM (n=59 per genotype).

Table 10 – Fasting Serum Adiponectin Concentrations (Based on Dietary Group) of Lean and *fa/fa* Zucker Rats fed CLA-containing or Control Diets for 8 Weeks (Adapted from Zirk, 2005)

	9-11	10-12	TOG	BIO	NCK	CTL
Serum Adiponectin (ng/mL)	4801 ± 242^{b,c}	5736 ± 326^{a}	5572 ± 326^{a,b}	5474 ± 405^{a,b}	5337 ± 356^{a,b,c}	4607 ± 234^{c}

There was a significant main effect of diet ($p=0.02$)
Means with different letters are significantly different ($p=0.02$; based on Duncan's Multiple Range Test).
Data are presented as mean ± SEM (n=19 or 20 per group).

Table 11 – Fasting Serum Leptin Concentrations of Lean and *fa/fa* Zucker Rats fed CLA-containing or Control Diets for 8 Weeks (Adapted from Zirk, 2005)

	9-11	10-12	TOG	BIO	NCK	CTL
Serum Leptin (ng/mL)						
Lean	5 ± 1	5 ± 1	4 ± 1	3 ± 0.5	4 ± 0.5	4 ± 0.6
fa/fa	128 ± 4	110 ± 5*‡	110 ± 5†	104 ± 5$^{\#‡}$	97 ± 6$^{\#‡}$	125 ± 6$^{\#‡}$

Data are presented as mean ± SEM, n=10 per group.
There was a significant genotype x diet interaction (p=0.0004).
* Significantly different from *fa/fa* CTL ($p<0.05$).
$^{\#}$ Significantly different from *fa/fa* CTL ($p<0.01$).
† Significantly different from *fa/fa* 9-11 ($p<0.05$).
‡ Significantly different from *fa/fa* 9-11 ($p<0.01$).

Table 12 – Fasting Serum Insulin, C-peptide, TAG, FFA, and AUCg of Lean and *fa/fa* Zucker Rats fed CLA-containing or Control Diets for 8 Weeks (Adapted from Diakiw, 2005)

	9-11	10-12	TOG	BIO	NCK	CTL	*Overall genotype mean*[1]
Serum Insulin (pmol/L)							
Lean	270 ± 45	228 ± 30	230 ± 25	217 ± 32	257 ± 31	242 ± 30	
fa/fa	4138 ± 454	2255 ± 156[#‡]	2104 ± 218[#‡]	2454 ± 183[#‡]	1935 ± 162[#‡]	3991 ± 597[#‡]	
Serum C-peptide (pmol/L)							
Lean	1153 ± 542	1077 ± 205	1111 ± 130	1059 ± 161	1129 ± 130	1069 ± 100	
fa/fa	10440 ± 1237	7403 ± 653[†]	6899 ± 848[*‡]	8164 ± 1051	6917 ± 638[*‡]	9902 ± 707	
Serum TAG (mmol/L)							
Lean	0.64 ± 0.05	0.59 ± 0.08	0.55 ± 0.06	0.56 ± 0.05	0.63 ± 0.07	0.50 ± 0.08	*0.58 ± 0.03*
fa/fa	4.01 ± 0.75	5.61 ± 0.90	4.18 ± 0.53	5.25 ± 0.45	5.00 ± 0.56	5.24 ± 1.09	*4.88 ± 0.30*[^]
Serum FFA (mmol/L)							
Lean	0.55 ± 0.03	0.55 ± 0.03	0.50 ± 0.05	0.52 ± 0.03	0.49 ± 0.03	0.51 ± 0.05	*0.52 ± 0.02*
fa/fa	1.27 ± 0.07	1.41 ± 0.09	1.31 ± 0.10	1.30 ± 0.11	1.15 ± 0.10	1.24 ± 0.05	*1.27 ± 0.04*[^]
AUCg (mmol/min/mL)							
Lean	611 ± 26	594 ± 16	616 ± 36	613 ± 12	586 ± 30	622 ± 25	
fa/fa	958 ± 52	849 ± 54[#]	784 ± 35[#‡]	803 ± 32[#†]	756 ± 34[#‡]	1059 ± 48	

Data are presented as mean ± SEM, n=10 per group.
[1] Genotype means are provided if only a significant effect of genotype was found (TAG and FFA).
There was a significant genotype x diet interaction for serum insulin ($p<0.0001$), C-peptide ($p=0.0161$) and AUCg (0.0006).
[#] Significantly different from *fa/fa* CTL ($p<0.01$).
[†] Significantly different from *fa/fa* 9-11 ($p<0.05$).
[‡] Significantly different from *fa/fa* 9-11 ($p<0.01$).
[^] Significant genotype difference ($p<0.0001$).

Summary:

Leptin

- Fasting serum leptin concentration was 96.8% higher in the *fa/fa* CTL rats than the lean CTL rats.
- There were no differences in serum leptin among the lean treatment groups.
- Compared to the *fa/fa* CTL group, serum leptin concentrations were 12.0% lower in the *fa/fa* 10-12 group, 11.7% lower in the *fa/fa* TOG group, 17.0% in the *fa/fa* BIO group, and 22.8% in the *fa/fa* NCK group.
- Serum leptin did not differ between the *fa/fa* 9-11 and the *fa/fa* CTL groups.

Adiponectin

- There was a significant effect of genotype on fasting serum adiponectin; however, contrary to what was expected, serum adiponectin in *fa/fa* rats was 25.8% higher than lean rats.
- There was also a significant effect of diet on serum adiponectin concentrations. Rats in the 10-12 groups had the highest concentrations, and this value was significantly higher than the CTL groups and the 9-11 groups (19.7% and 16.3% higher, respectively). There were no significant differences among the other diet groups.

Insulin

- Fasting serum insulin concentration was 93.9% higher in the *fa/fa* CTL rats than the lean CTL rats.
- There were no differences among the dietary treatment groups in the lean genotype; however, differences were found among the dietary treatment groups in the *fa/fa* genotype.
- Compared to the *fa/fa* CTL group, serum insulin concentrations were 43.5% lower in the *fa/fa* 10-12 group, 47.3% lower in the *fa/fa* TOG group, 38.5% lower in the *fa/fa* BIO group, and 51.5% lower in the *fa/fa* NCK group.
- There was no difference in serum insulin between the *fa/fa* 9-11 and *fa/fa* CTL groups.

C-peptide

- Fasting serum C-peptide was 89.2% higher in the *fa/fa* CTL rats than the lean CTL rats.
- There were no differences among the dietary treatment groups in the lean genotype; however, differences were found among the dietary treatment groups in the *fa/fa* genotype.
- Compared to the *fa/fa* CTL group, serum C-peptide concentrations were 30.3% lower in the *fa/fa* TOG group and 30.1% lower in the *fa/fa* NCK group.

- Although the *fa/fa* 10-12 group was 25.2% lower and the *fa/fa* BIO group was 17.0% lower than *fa/fa* CTL, these differences did not reach statistical significance.
- There was no statistical difference between the *fa/fa* CTL group and the *fa/fa* 9-11 group. However, serum C-peptide concentrations in the *fa/fa* 10-12 group were significantly lower (29.0% lower) than the *fa/fa* 9-11 group.

TAG

- Fasting serum TAG concentrations in the fa/fa rats was 89.6% higher than the lean rats.
- None of the CLA-containing diets affected serum TAG in lean or *fa/fa* rats.
- Although the average serum TAG concentration of the *fa/fa* 10-12 group was 28.5% higher than the *fa/fa* 9-11 group and 25.5% higher than the *fa/fa* TOG group, these differences did not reach statistical significance.

FFA

- Fasting serum FFA concentration in the *fa/fa* rats was 59% higher than lean rats.
- None of the CLA-containing diets affected serum FFA in either lean or *fa/fa* rats.

AUCg

- AUCg for the *fa/fa* rats was 41.3% higher than AUCg in the lean rats.
- There were no differences in AUCg among treatment groups in the lean genotype.
- Compared to *fa/fa* CTL, AUCg was 19.8% lower in the *fa/fa* 10-12 group, 26.0% lower in the *fa/fa* TOG group, 24.2% lower in the *fa/fa* BIO group, and 28.7% lower in the *fa/fa* NCK group.
- The *fa/fa* 9-11 and *fa/fa* CTL groups did not differ in respect to AUCg.
- Although AUCg of the *fa/fa* 10-12 group was 11.4% lower than the *fa/fa* 9-11 group, this difference did not reach statistical significance, while the differences among the *fa/fa* 9-11 group and the *fa/fa* TOG, *fa/fa* BIO, and *fa/fa* NCK groups did reach statistical significance.

RESULTS

STUDY 1

Baseline Analysis

Body Weight

There was a significant genotype x time interaction for differences in body weight. *Fa/fa* baseline rats weighed 23.9% more than lean baseline rats, while *fa/fa* endpoint rats weighed 41.2% more than lean endpoint rats (Figure 4). Due to normal growth, lean endpoint rats weighed 60.4% more than lean baseline rats. Body weight was 69.5% higher in *fa/fa* endpoint rats than *fa/fa* baseline rats.

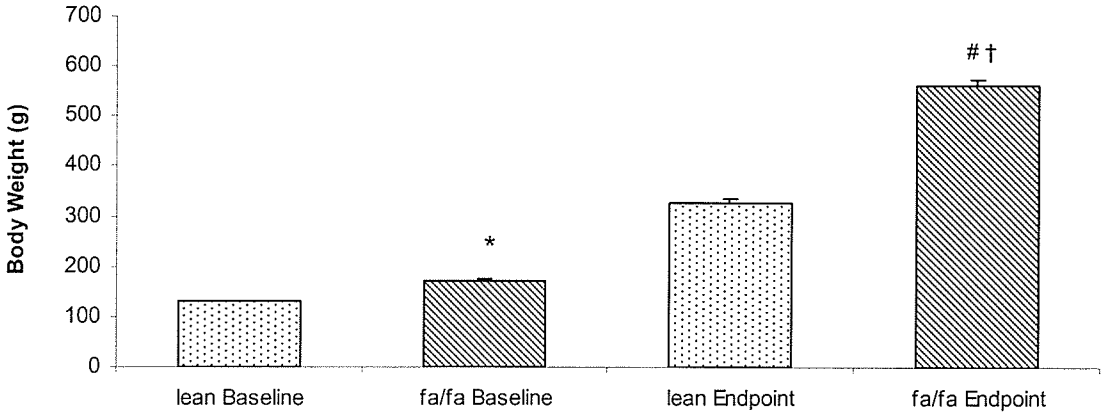


Figure 4. Body weights of lean and endpoint Zucker rats. There was a significant genotype x time interaction ($p<0.0001$). The * denotes a significant difference from lean baseline ($p<0.001$). The # denotes a significant difference from *fa/fa* baseline ($p<0.001$). The † denotes a significant difference from lean endpoint ($p<0.001$). Data are presented as means ± SEM (n=10 per group).

Absolute Liver Weight

There was a significant genotype x time interaction for differences in liver weight. While liver weight was already 40.5% higher in *fa/fa* baseline rats compared to lean baseline rats, liver weight of *fa/fa* endpoint rats was 61.1% higher than lean endpoint rats (Figure 5). Lean endpoint rats had livers that were 47.2% heavier than lean baseline rats, while *fa/fa* endpoint rats had livers that were 65.4% higher than *fa/fa* endpoint rats.

Adjusted Liver Weight

Only a significant effect of genotype was found when liver weight was adjusted for body weight, with the *fa/fa* rats having significantly higher adjusted liver weights than lean rats (Figure 6). A significant effect of genotype signifies that the mean of all lean rats (baseline and endpoint combined) is significantly different that all *fa/fa* rats (baseline and endpoint combined); therefore, for Figure 6 and all subsequent graphs of parameters with significant main effects of genotype and time, in order to display the means of all four groups, symbols are replaced with the significance statement placed above the graph. Genotype means can be found in Appendix 1, table A1-1.

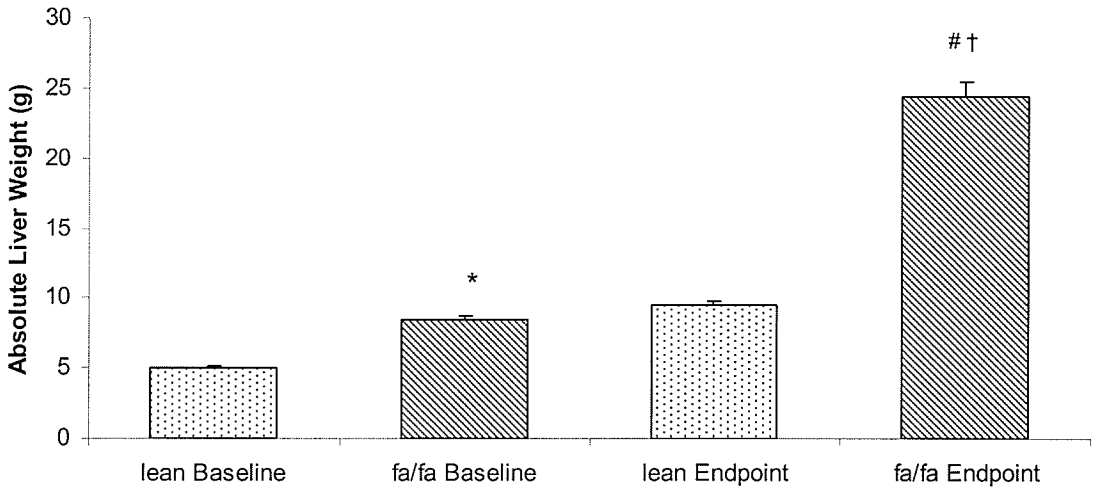


Figure 5. Absolute liver weights of baseline and endpoint Zucker rats. There was a significant genotype x time interaction ($p<0.0001$). The * denotes a significant difference from lean baseline ($p<0.001$). The # denotes a significant difference from *fa/fa* baseline ($p<0.001$). The † denotes a significant difference from lean endpoint ($p<0.001$). Data are presented as means ± SEM (n=10 per group).

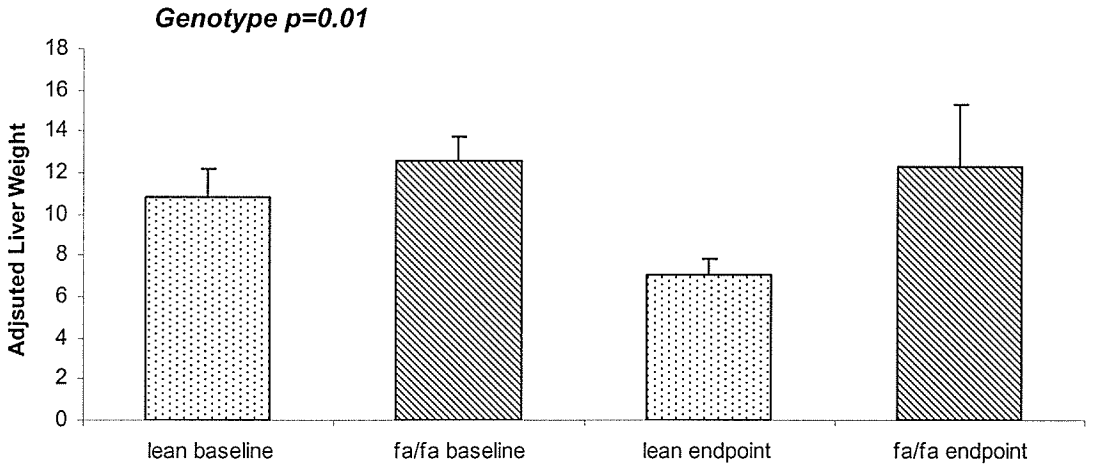


Figure 6. Adjusted liver weights of baseline and endpoint Zucker rats. There was a significant effect of genotype ($p=0.01$). Data are presented as adjusted means ± SEM (n=10 per group), where liver weight was adjusted for body weight using ANCOVA.

Fasting Serum Insulin and C-peptide

There were significant effects of genotype and time on fasting serum insulin concentrations, but no significant genotype x time interaction. At 6 weeks of age, serum insulin was already 11.7-fold higher in the baseline *fa/fa* Zucker rats compared to the baseline lean Zucker rats. As expected, serum insulin in the *fa/fa* endpoint rats was 16.4-fold higher than that of lean endpoint rats at 14 weeks of age.

Comparing the effect of time, serum insulin of lean endpoint rats was approximately 55.6% higher than lean baseline rats. Similarly, serum insulin of *fa/fa* endpoint rats was approximately 68.3% higher than that of *fa/fa* baseline rats (Figure 7).

There was a significant genotype x time interaction for differences in fasting serum C-peptide concentrations. At both baseline and the end of the study, *fa/fa* Zucker rats had higher serum C-peptide (91.4% and 89.2% higher, respectively). While there was no difference between lean baseline rats and lean endpoint rats, serum C-peptide was 65.8% higher in *fa/fa* endpoint rats compared to *fa/fa* baseline rats (Figure 8).

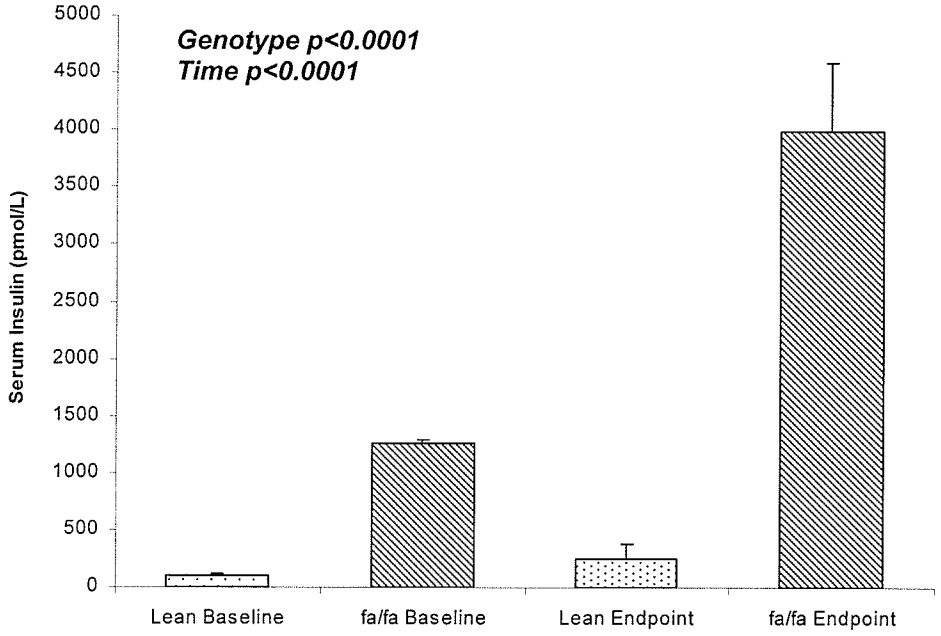


Figure 7. Fasting serum insulin concentrations of baseline and endpoint Zucker rats. There was a significant effect of genotype ($p<0.001$) and time ($p<0.001$). Statistics are based on log transformed data. Data are presented as means ± SEM (n=9 or 10 per group).

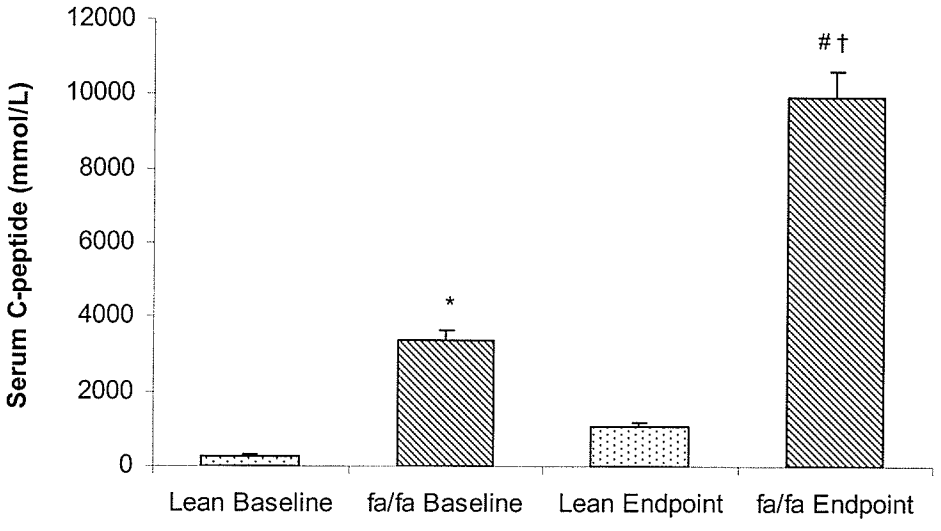


Figure 8. Fasting serum C-peptide concentrations of baseline and endpoint Zucker rats. There was a significant genotype x time interaction ($p<0.0001$). The * denotes a significant difference from lean baseline ($p<0.001$). The # denotes a significant difference from *fa/fa* baseline ($p<0.001$). The † denotes a significant difference from lean endpoint ($p<0.001$). Data are presented as means ± SEM (n=10 per group).

Hepatic Lipid Concentration

There was a significant genotype x time interaction for differences in hepatic lipid content. The *fa/fa* baseline rats had a 51.3% higher hepatic lipid concentration compared to lean baseline rats. Hepatic lipid concentration was 71.2% higher in *fa/fa* endpoint rats than lean endpoint rats. There was no significant difference in hepatic lipid concentration between the lean baseline and endpoint rats; however, hepatic lipid concentration was 45.9% higher in the *fa/fa* endpoint rats compared to the *fa/fa* baseline rats (Figure 9).

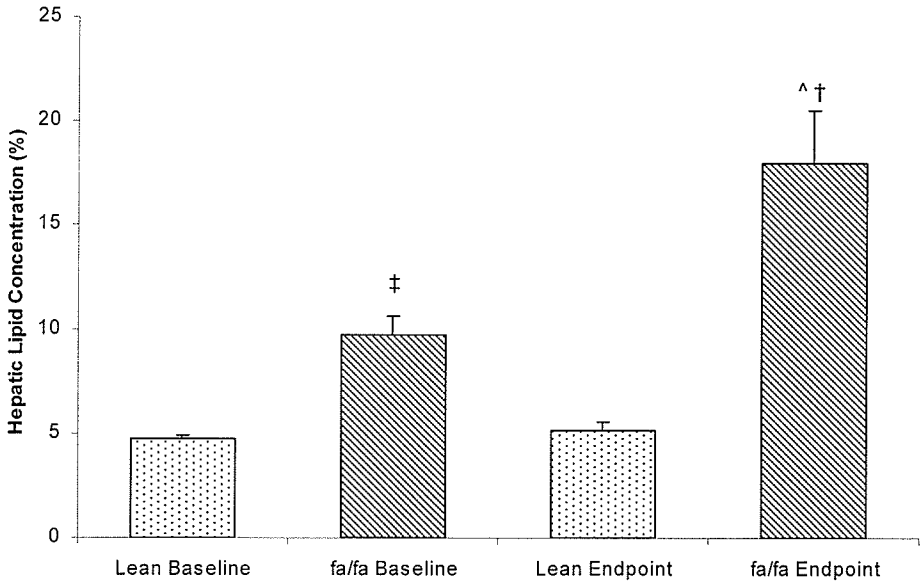


Figure 9. Hepatic lipid concentration of baseline and endpoint Zucker rats. There was a significant genotype x time interaction ($p=0.0156$). The ‡ denotes a significant difference from lean baseline ($p<0.05$). The ^ denotes a significant difference from *fa/fa* baseline ($p<0.05$). The † denotes a significant difference from lean endpoint ($p<0.001$). Statistics are based on log transformed data. Data are presented as means ± SEM (n=4, 6, or 10 per group).

Fasting Serum TAG and FFA

Only a significant effect of genotype was found for serum TAG between baseline and endpoint rats. Serum TAG concentrations were 88.9% higher in *fa/fa* baseline rats compared to lean baseline rats, and were 90.6% higher in *fa/fa* endpoint rats compared to lean endpoint rats (Figure 10).

Serum TAG concentrations of *fa/fa* baseline and *fa/fa* endpoint rats were almost identical (5.55 ± 0.45 mmol/L and 5.24 ± 1.09 mmol/L, respectively). Serum TAG concentrations of lean baseline and lean endpoint rats were also similar (0.494 ± 0.079 mmol/L in lean endpoint vs. 0.620 ± 0.050 mmol/L in lean baseline), and were not statistically different.

There was a significant genotype x time interaction for the differences in serum FFA concentrations. Interestingly, serum FFA were higher in the lean baseline rats than they were in the lean endpoint rats. There was no difference between the serum FFA in the *fa/fa* baseline rats and the *fa/fa* endpoint rats. Serum FFA of the *fa/fa* baseline rats were 35.3% higher than the lean baseline rats, while they were 58.7% higher in the *fa/fa* endpoint rats compared to the lean endpoint rats (Figure 11).

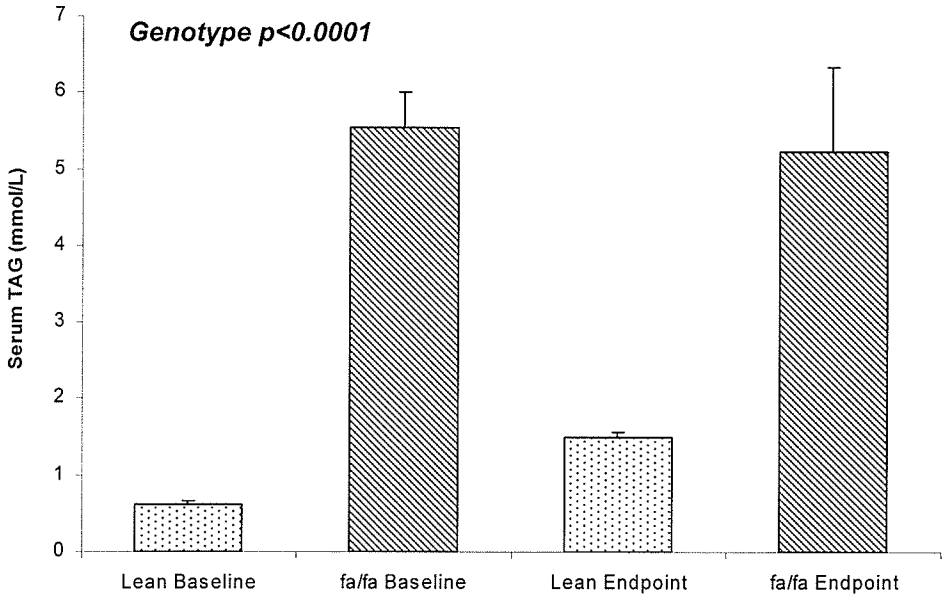


Figure 10. Fasting serum TAG concentrations of baseline and endpoint Zucker rats. There was a significant effect of genotype ($p<0.0001$). Data are presented as means ± SEM (n=10 per group).

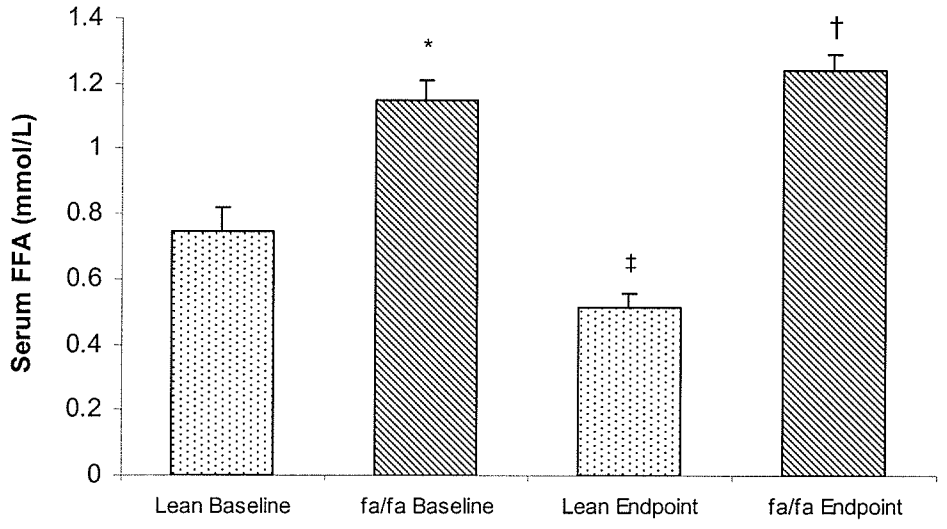


Figure 11. Fasting serum FFA concentrations of baseline and endpoint Zucker rats. There was a significant genotype x time interaction ($p=0.0095$). The * denotes a significant difference from lean baseline ($p<0.001$). The ‡ denotes a significant difference from lean baseline ($p<0.05$). The † denotes a significant difference from lean endpoint ($p<0.001$). Data are presented as means ± SEM (n=10 per group).

Fasting Serum Adiponectin

A significant genotype x time interaction was found for differences in serum adiponectin. Serum adiponectin of *fa/fa* baseline rats was 38.9% higher than the lean baseline rats (Figure 12). Comparing the *fa/fa* baseline and *fa/fa* endpoint groups, serum adiponectin was slightly higher in the baseline group, but this was not statistically significant. Likewise, although serum adiponectin was 20.5% higher in the *fa/fa* endpoint rats than the lean endpoint rats, this was not significant. There was no statistical difference between the lean baseline and lean endpoint rats (Figure 12).

Serum ALT

There was a significant genotype x time interaction for the differences in serum ALT. Serum ALT was significantly higher in *fa/fa* endpoint rats than in lean endpoint rats and in *fa/fa* baseline rats (86.2% and 68.9%, respectively). In baseline rats, serum ALT was 55.0% higher in *fa/fa* rats compared to lean rats. Lean baseline and lean endpoint rats did not differ with respect to serum ALT (Figure 13).

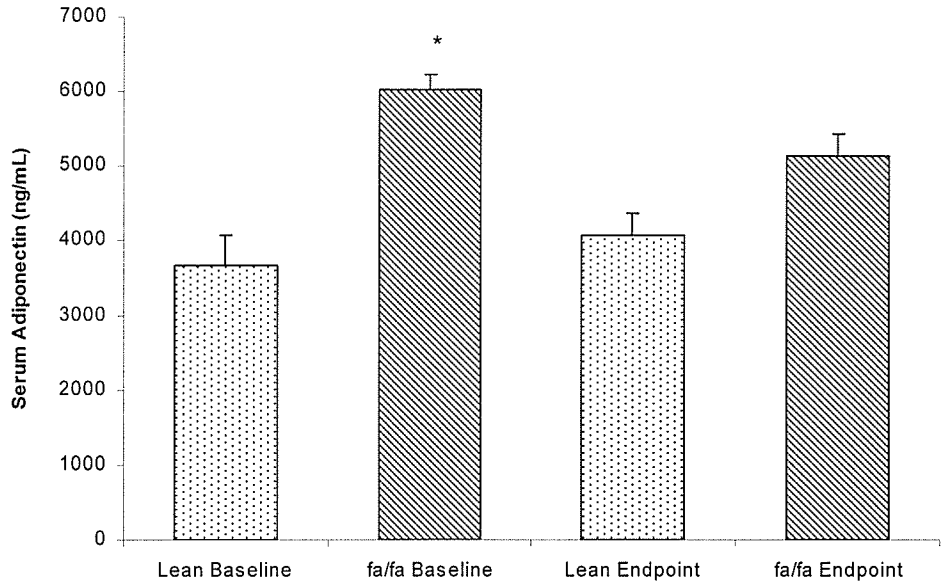


Figure 12. Serum adiponectin concentrations of baseline and endpoint Zucker rats. There was a significant genotype x time interaction (p=0.0092). The * denotes a significant difference from lean baseline (p<0.001). Data are presented as mean ± SEM (n=8, 9, or 10 per group).

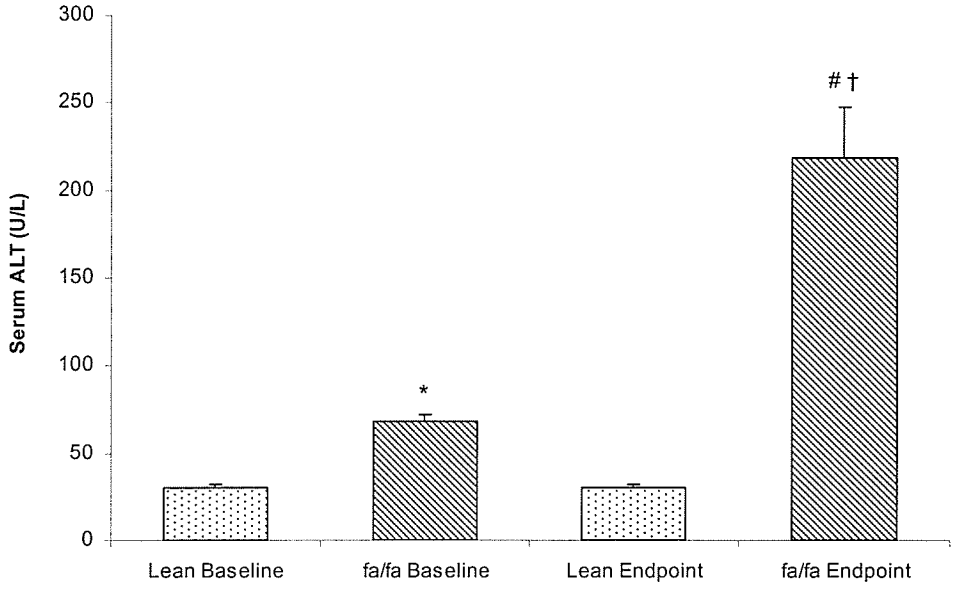


Figure 13. Serum ALT concentrations of baseline and endpoint Zucker rats. There was a significant genotype x time interaction (p<0.0001). The * denotes a significant difference from lean baseline (p<0.001). The # denotes a significant difference from *fa/fa* baseline (p<0.001). The † denotes a significant difference from lean endpoint (p<0.001). Data is presented as means ± SEM (n=10 per group).

Dietary CLA Analysis

Total Feed Intake and Final Body Weights

At the end of the study, lean rats had a lower total feed intake than the *fa/fa* rats (Figure 14). There was a significant genotype x diet interaction for differences in total feed intake. Total feed intake of the fa 10-12 group was 16.0% and 8.3% lower compared to the fa CTL and fa 9-11 groups, respectively. Rats in the fa BIO and fa NCK group had 9.0% and 12% lower feed intakes, respectively, compared to the fa CTL group. Feed intake did not differ among the lean groups.

As expected, final body weights of the *fa/fa* rats were 1.7-fold higher than the lean rats (Figure 15). There were no differences in final body weight among treatment groups in both genotypes; therefore, PW groups were not included in subsequent analyses.

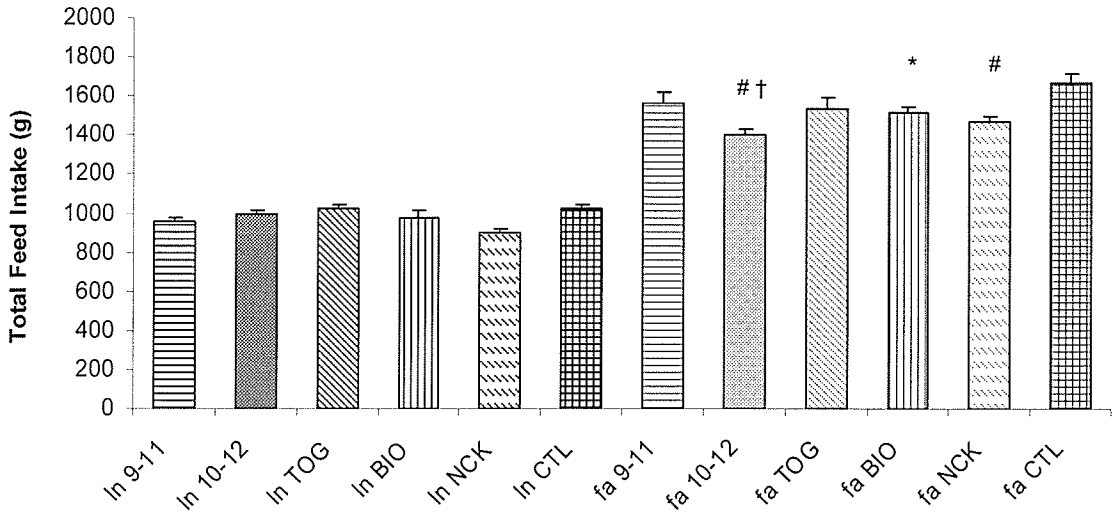


Figure 14. Total feed intake of lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. There was a significant genotype x diet interaction ($p<0.0166$). The * denotes a significant difference from fa CTL ($p<0.05$). The # denotes a significant difference from fa CTL ($p<0.01$). The † denotes a significant difference from the fa 9-11 group ($p<0.05$). Data are presented as means ± SEM (n=10 per group).

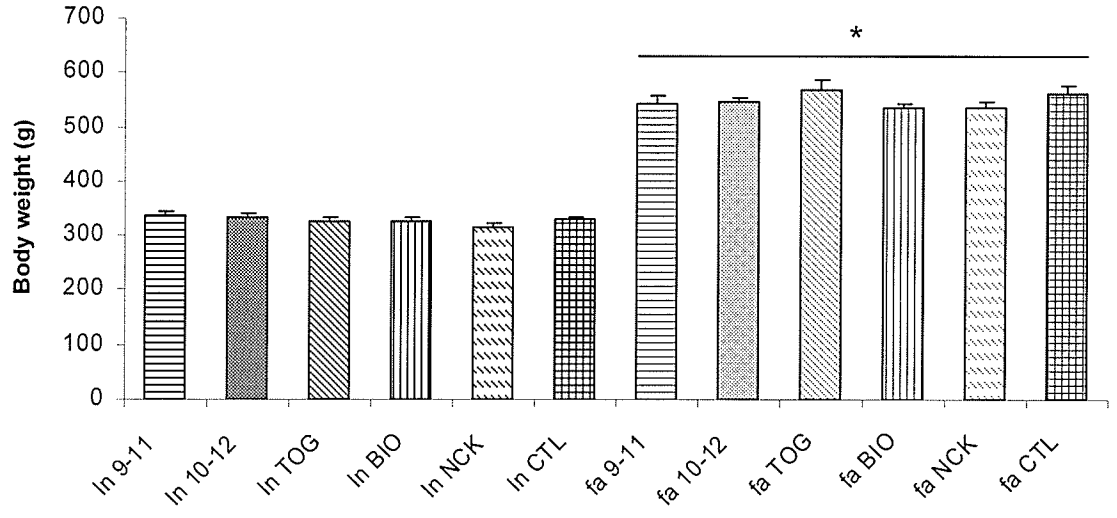


Figure 15. Final body weights of lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. The * denotes a significant genotype effect ($p<0.0001$). Data are presented as means ± SEM (n=10 per group).

Absolute and Adjusted Liver Weights

A significant genotype x diet interaction was detected for differences in absolute liver weights. Compared to fa CTL, absolute liver weights were 28.6%, 29.4%, 28.2%, and 28.6% lower in the fa 10-12, fa TOG, fa BIO, and fa NCK groups, respectively. There was no significant difference between the fa 9-11 and fa CTL groups in regards to liver weight. Liver weights did not differ among dietary groups in the lean genotype (Figure 16).

Significant genotype x diet interactions were found for the differences in adjusted liver weights, with a similar pattern as seen with absolute liver weights. Significantly lower adjusted liver weights were observed in the fa 10-12 (32.0% reduction), fa TOG (36.4% reduction), fa BIO (29.5% reduction), and fa NCK (30.0% reduction) groups compared to the fa CTL group. Adjusted liver weight of the fa 9-11 group did not differ from the fa CTL group. There were no differences among treatment groups in the lean genotype (Figure 17).

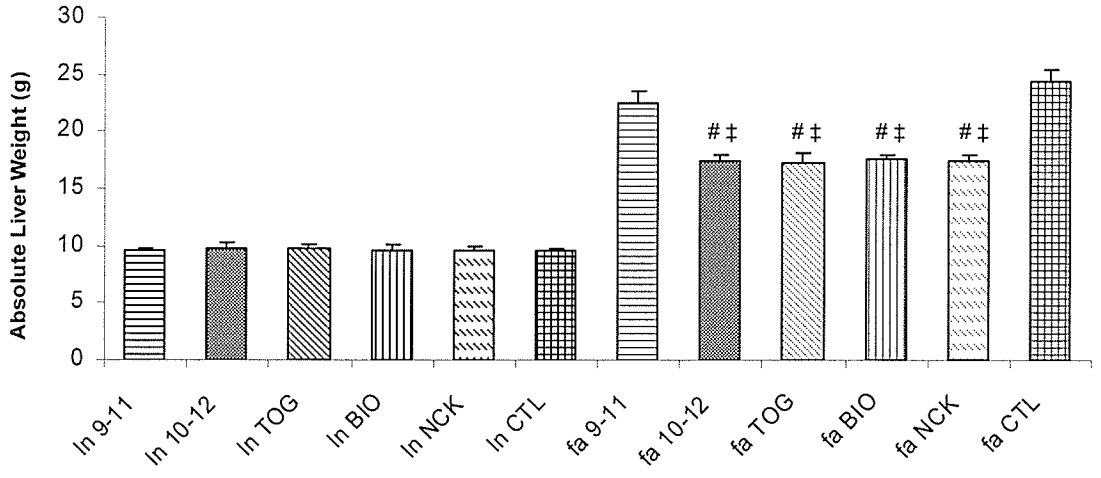


Figure 16. Absolute liver weights of lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. There was a significant genotype x diet interaction ($p<0.0001$). The # denotes a significant difference from the fa CTL group ($p<0.01$). The ‡ symbol denotes a significant difference from the fa 9-11 group ($p<0.01$). Data are presented as means ± SEM (n=10 per group).

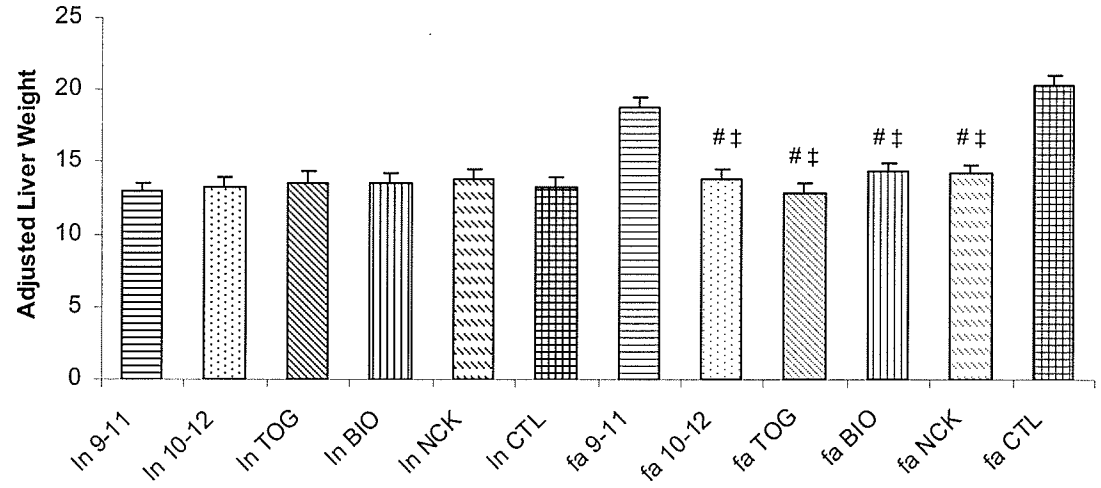


Figure 17. Adjusted liver weights of lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. There was a significant genotype x diet interaction ($p<0.0001$). The # denotes a significant difference from the fa CTL group ($p<0.01$). The ‡ symbol denotes a significant difference from the fa 9-11 group ($p<0.01$). Data are presented as adjusted means ± SEM (n=10 per group), where liver weight was adjusted for body weight using ANCOVA.

Hepatic Lipid Concentration

A significant genotype x diet interaction was detected for the differences in hepatic lipid concentration. Hepatic lipid concentration in the fa 9-11 group was not different from the fa CTL group. However, dramatic reductions were observed in the fa 10-12, fa TOG, fa BIO, and fa NCK groups. Compared to the fa CTL group, hepatic lipid concentration was 65.3%, 63.3%, 50.1%, and 66.7% lower in the fa 10-12, fa TOG, fa BIO, and fa NCK groups, respectively. There were no differences among the lean treatment groups (Figure 18).

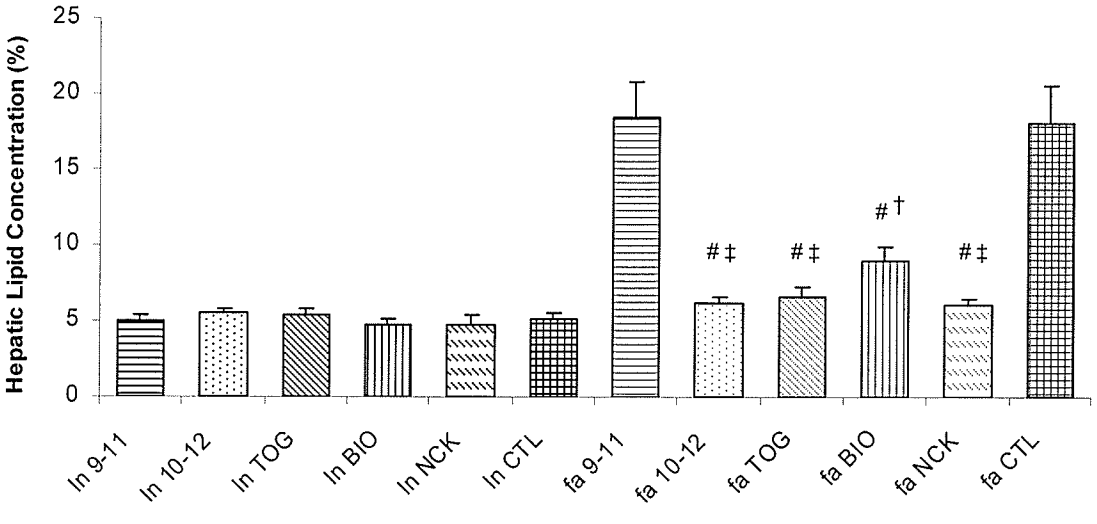


Figure 18. Hepatic lipid concentration of lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. There was a significant genotype x diet interaction ($p<0.0004$). The # denotes a significant difference from fa CTL ($p<0.01$). The ‡ denotes a significant difference from fa 9-11 ($p<0.01$). The † denotes a significant difference from the fa 9-11 group ($p<0.05$). Data are presented as means ± SEM (n=4 per group).

Fasting Serum Cholesterol

A significant genotype x diet interaction was detected for the differences in fasting serum cholesterol. Rats in the fa 10-12, fa TOG, fa BIO, and fa NCK groups had significantly lower serum cholesterol (30.0%, 24.7%, 26.9%, and 23.6% lower, respectively) compared to rats in the fa CTL group (Figure 19). Serum cholesterol concentrations of the fa 9-11 and the fa CTL groups were not different. There were no differences in serum cholesterol among the lean treatment groups.

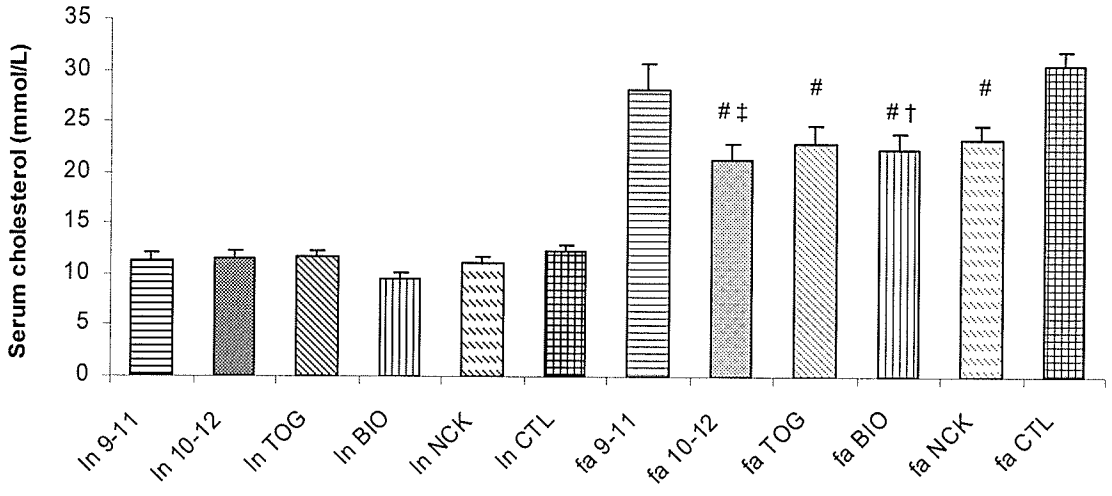


Figure 19. Fasting serum cholesterol concentrations of lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. There was a significant genotype x diet interaction ($p=0.0114$). The # denotes a significant difference from fa CTL ($p<0.01$). The ‡ denotes a significant difference from fa 9-11 ($p<0.01$). The † denotes a significant difference from fa 9-11 ($p<0.05$). Data are presented as means ± SEM (n=10 per group).

Fasting Serum Glucose

The *fa/fa* rats had a slightly but significantly higher (19.6%) fasting serum glucose compared to the lean rats (figure 20). There was no significant difference in fasting glucose among any of the dietary treatment groups in either genotype.

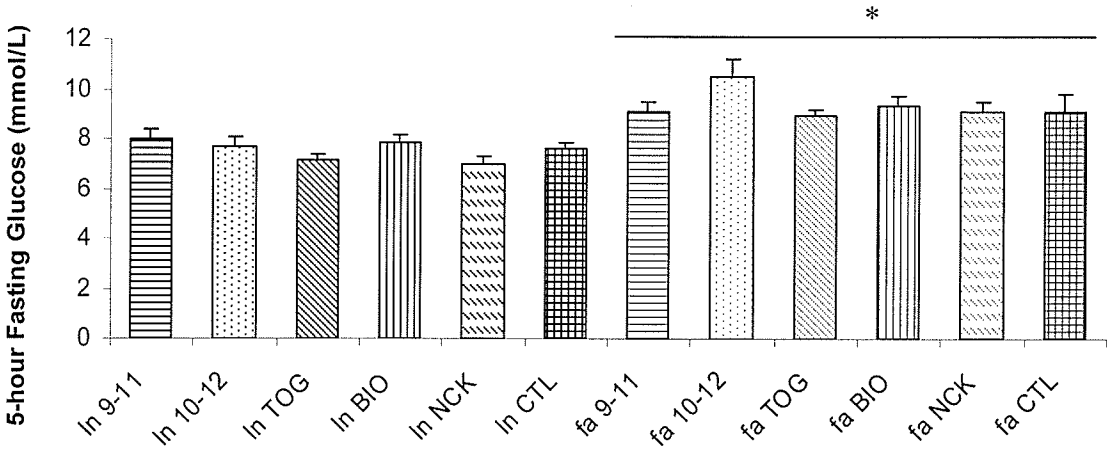


Figure 20. Five-hour fasting serum glucose concentrations of lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. The * denotes a significant genotype effect ($p<0.0001$). Data are presented as means ± SEM (n=10 per group).

Serum ALT

Overall, lean rats had a significantly lower serum ALT concentration than the *fa/fa* rats (35.8 U/L vs. 140 U/L, respectively). A significant genotype x diet interaction was detected for differences in serum ALT concentrations. Within the *fa/fa* genotype, there was no significant difference between the fa CTL and the fa 9-11 groups; however, serum ALT was 49.6% lower in the fa 10-12 group, 45.1% lower in the fa TOG groups, 48.9% lower in the fa BIO group, and 49.5% lower in the fa NCK group, compared to the fa CTL group (Figure 21). Dietary treatment groups in the lean genotype did not differ with respect to serum ALT concentrations.

Serum Haptoglobin

Overall, serum haptoglobin was 57.7% higher in the *fa/fa* rats than the lean rats. A significant genotype x diet interaction was detected for serum haptoglobin. Compared to fa CTL, serum haptoglobin concentrations were 30.4%, 24.0%, 23.0%, and 29.9% lower in the fa 10-12, fa TOG, fa BIO, and fa NCK groups, respectively (Figure 22). Although serum haptoglobin was slightly higher in the fa 9-11 group compared to the fa CTL group, this difference was not significant. There were no differences in serum haptoglobin among the dietary treatment groups in the lean genotype.

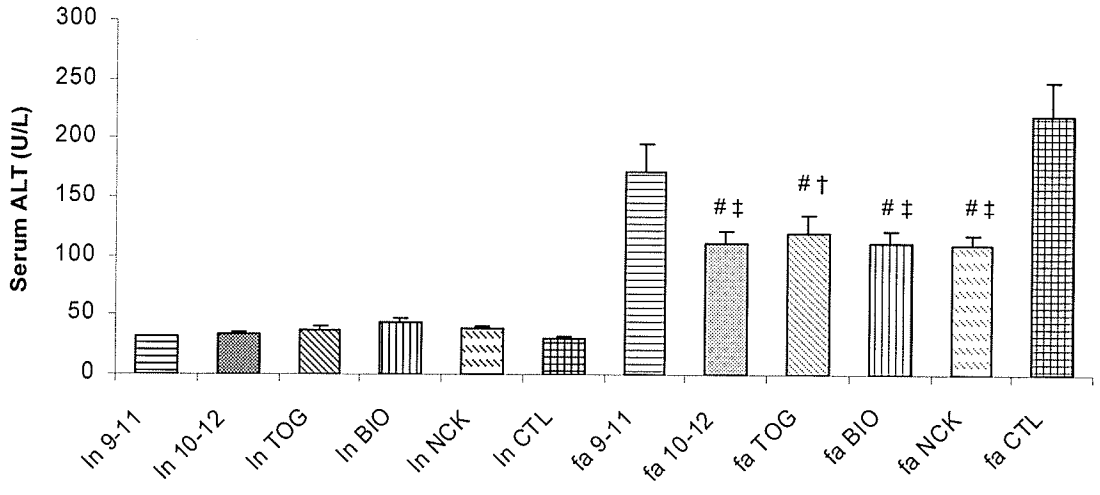


Figure 21. Serum ALT concentrations of lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. There was a significant genotype x diet interaction ($p<0.0001$). The # denotes a significant difference from fa CTL ($p<0.01$). The ‡ denotes a significant difference from fa 9-11 ($p<0.01$). The † denotes a significant difference from fa 9-11 ($p<0.05$). Data are presented as means ± SEM (n=10 per group).

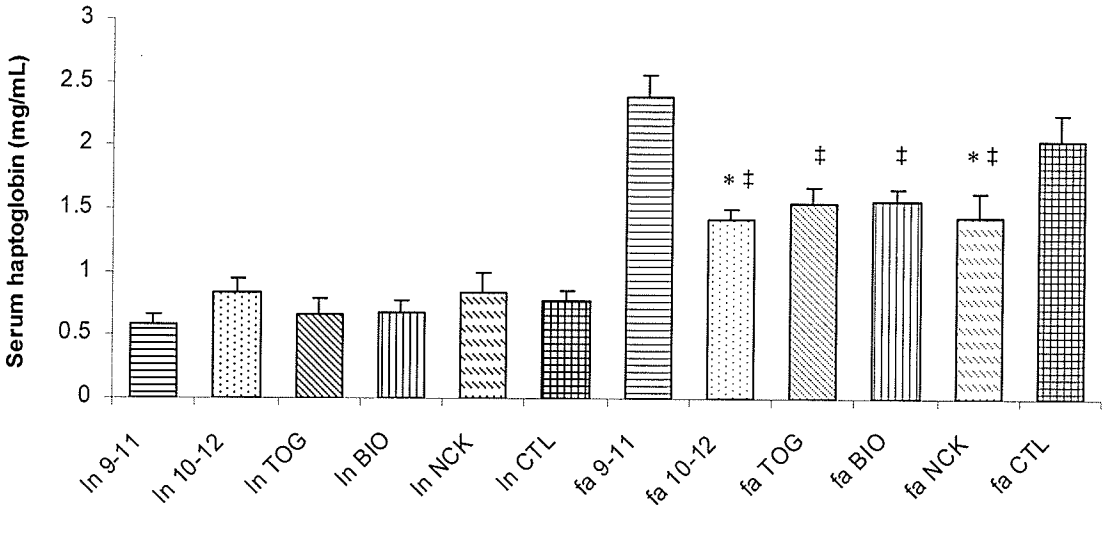


Figure 22. Serum haptoglobin concentrations of lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. There was a significant genotype x diet interaction ($p<0.0001$). The * denotes a significant difference from fa CTL ($p<0.05$). The ‡ denotes a significant difference from fa 9-11 ($p<0.01$). Data are presented as means ± SEM (n=8 per group).

Hepatic Proteins

i. AMPK

There was a significant effect of genotype on hepatic AMPK protein levels, with the lean rats having approximately 32.4% more AMPK (Figure 23). In both genotypes, AMPK protein levels did not differ among dietary treatment groups.

ii. pAMPKα

Overall, lean and *fa/fa* Zucker rats did not differ in respect to pAMPKα protein levels (Figure 24). There were no differences in pAMPKα protein levels among dietary treatment groups in either genotype.

iii. Full-length SREBP-1 (SREBP-1 p125)

There was a significant effect of genotype on protein levels of hepatic SREBP-1 p125, with the *fa/fa* rats having slightly lower SREBP-1 p125 (Figure 25). In both genotypes, hepatic SREBP-1 p125 protein levels did not differ among the dietary treatment groups.

iv. SREBP-1 p68

Overall, lean and *fa/fa* Zucker rats did not differ with respect to hepatic SREBP-1 p68 protein levels (Figure 26). There were no differences in SREBP-1 p68 levels among dietary treatment groups in either genotype.

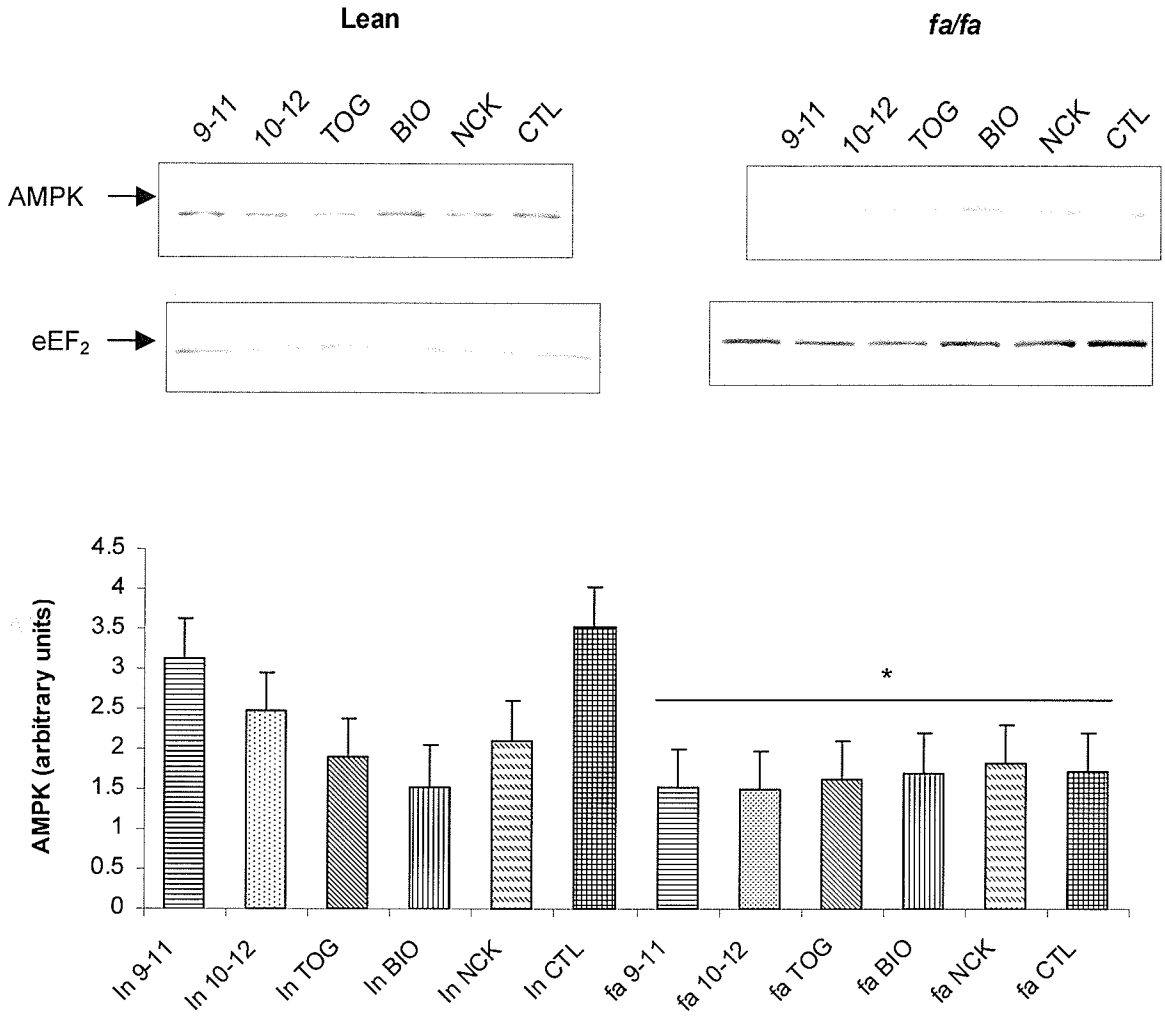


Figure 23. Hepatic AMPK protein levels of lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. The * denotes a significant genotype effect (p=0.0127). Data are presented as adjusted means ± SEM (n=4 per group), where AMPK values were adjusted for intensity and loading controls using ANCOVA.

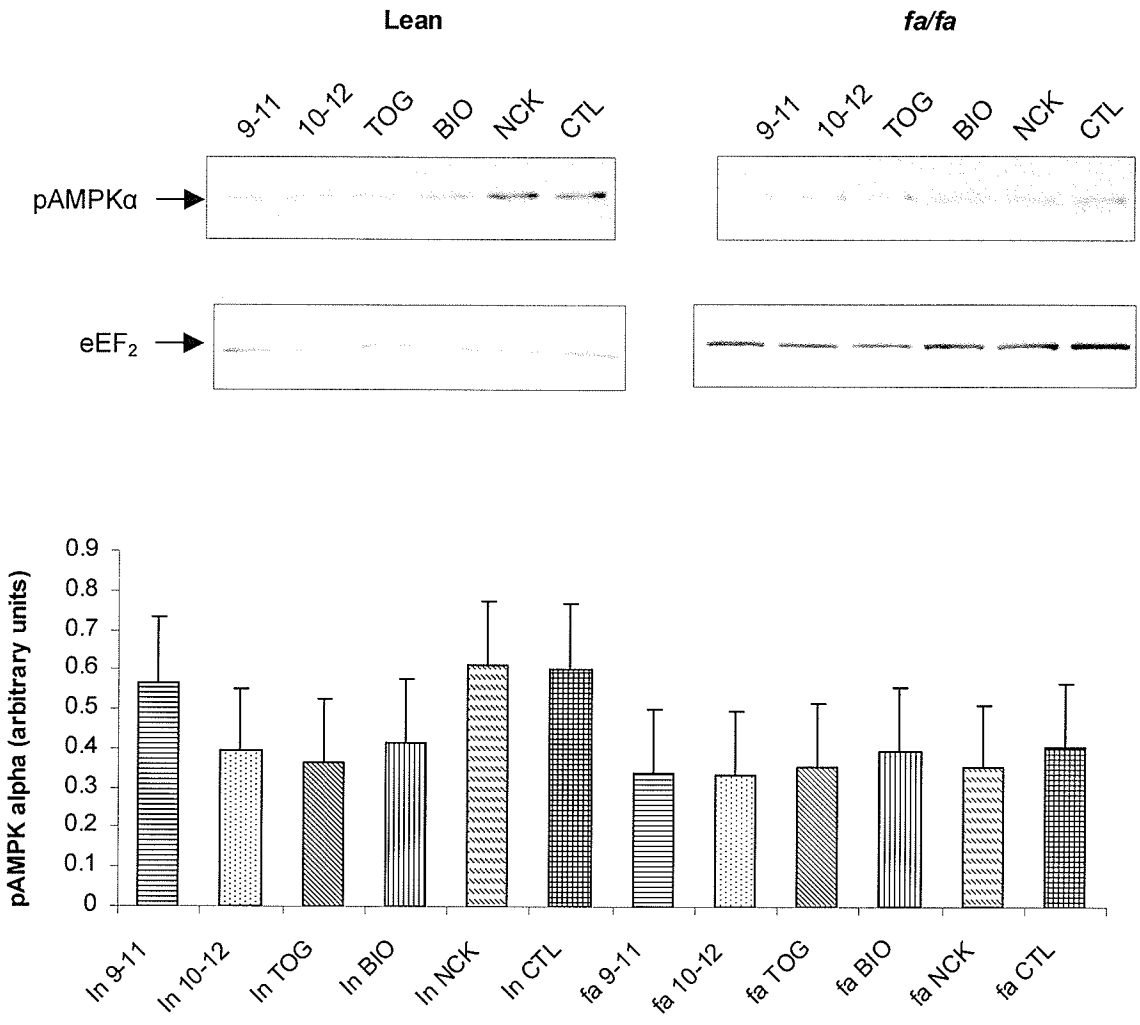


Figure 24. Hepatic pAMPKα protein levels in lean and *fa/fa* Zucker rats fed CLA isomers for 8 weeks. There were no genotype, diet, or genotype x diet differences among the groups. Data are presented as adjusted means ± SEM (n=4 per group), where pAMPKα values were adjusted for intensity and loading controls and total hepatic AMPK using ANCOVA.

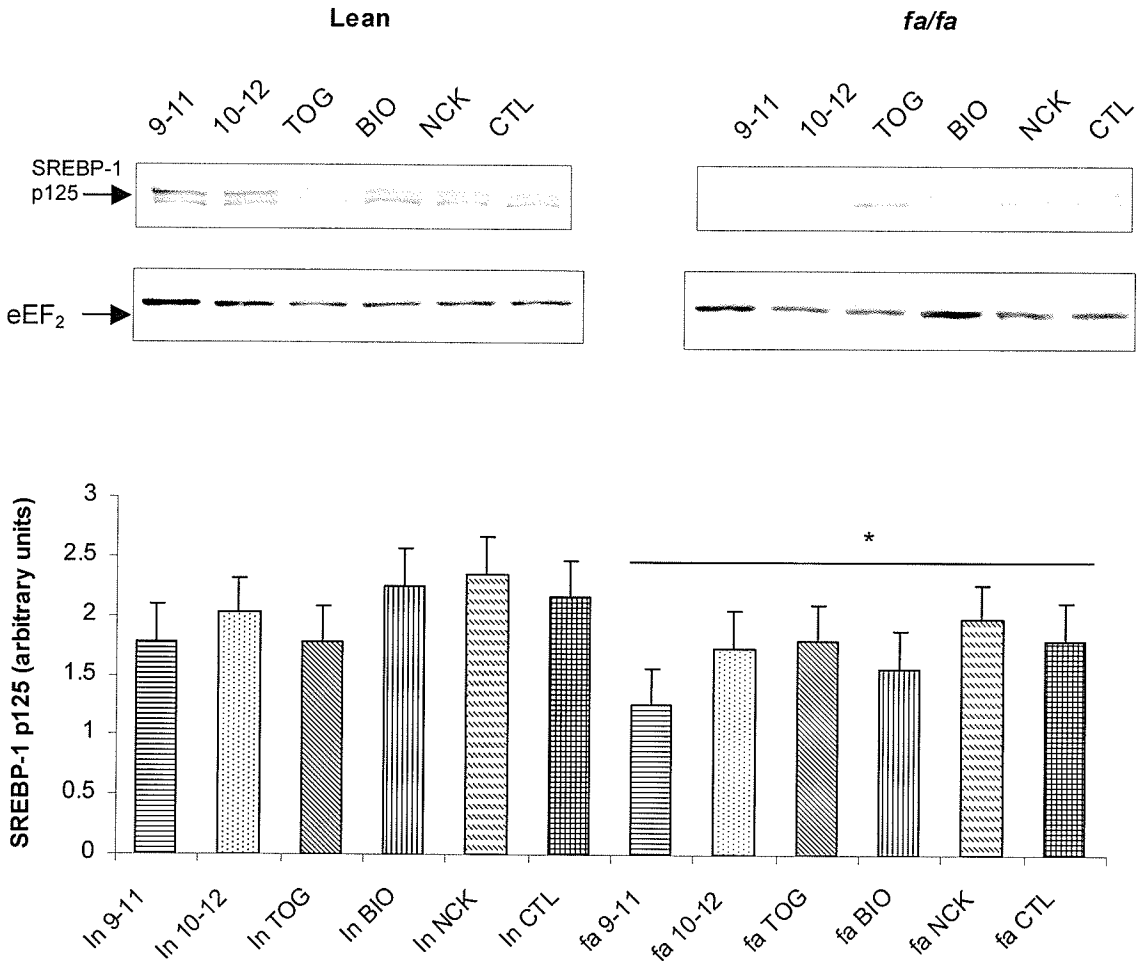


Figure 25. Hepatic SREBP-1 p125 protein levels in lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. The * denotes a significant genotype effect (p=0.05). Data are presented as adjusted means ± SEM (n=4 per group), where SREBP-1 p125 values were adjusted for intensity and loading controls using ANCOVA.

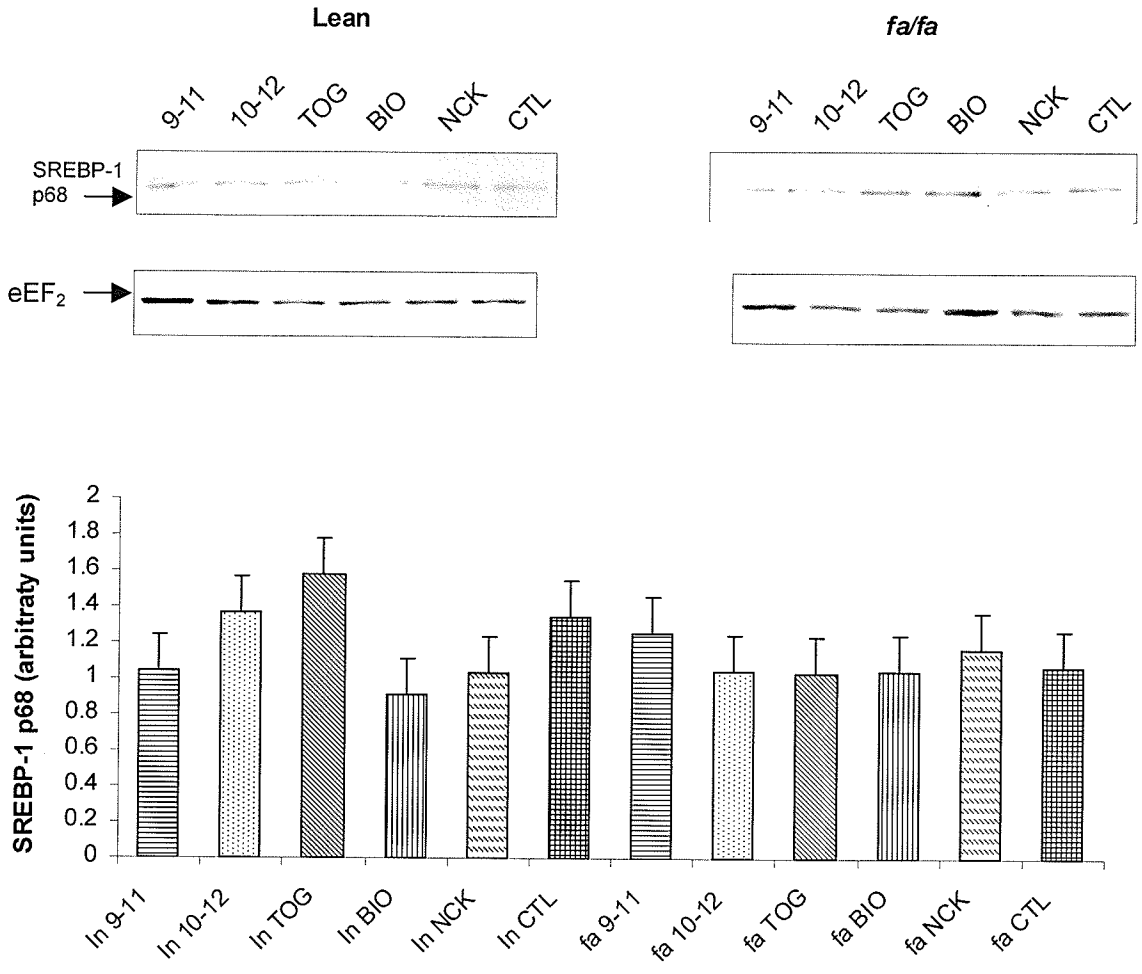


Figure 26. Hepatic SREBP-1 p68 protein levels in lean and *fa/fa* Zucker rats fed CLA isomers or control diet for 8 weeks. There were no significant genotype, diet, or genotype x diet differences among the groups. Data are presented as adjusted means ± SEM (n=4 per group), where SREBP-1 p68 values were adjusted for intensity and loading controls and total hepatic SREBP-1 using ANCOVA.

Correlations

After examining the data, it appeared that the beneficial effects elicited by the 10-12, TOG, BIO and NCK diets in *fa/fa* rats were following a similar pattern in some of the parameters that were measured. Therefore, correlative statistics were used to analyze the relationship between some of hepatic-centred parameters that were following the same pattern. In particular, insulin was paired with hepatic lipid concentration, ALT and haptoglobin to quantify the relationship between improved insulin resistance and hepatic steatosis, liver function, and a marker of the hepatic response to inflammatory stimuli. The following table provides r-values and p-values for the parameters that were examined.

Table 13 – r-values and p-values for Correlative Statistics

	r-value	p-value
Insulin – Hepatic Lipid Concentration	0.74	<0.0001
Insulin – ALT	0.79	<0.0001
Insulin – Haptoglobin	0.79	<0.0001
ALT – Hepatic Lipid Concentration	0.68	<0.0001
Adiponectin – Hepatic Lipid Concentration	0.10	0.5089
Adiponectin – ALT	0.27	0.03
Leptin – Haptoglobin	0.79	<0.0001
Leptin – Hepatic Lipid Concentration	0.62	<0.0001

STUDY 2 – FED STATE

Total Feed Intake

As expected, total feed intake was lowest in the ln CTL fed group (Figure 27). There were no differences in total feed intake among the *fa/fa* treatment groups.

Re-feed Intake

In Study 2, rats in the fa 9-11 fed, fa 10-12 fed, fa CTL fed, and ln CTL fed groups were exposed to a 2-hour re-feeding period prior to tissue collection. Two-hour re-feed intake did not differ among the re-fed groups (Figure 28).

Final Body Weight

Similar to Study 1, body weight was lowest in the ln CTL fed group (Figure 29), and there were no differences among the *fa/fa* treatment groups.

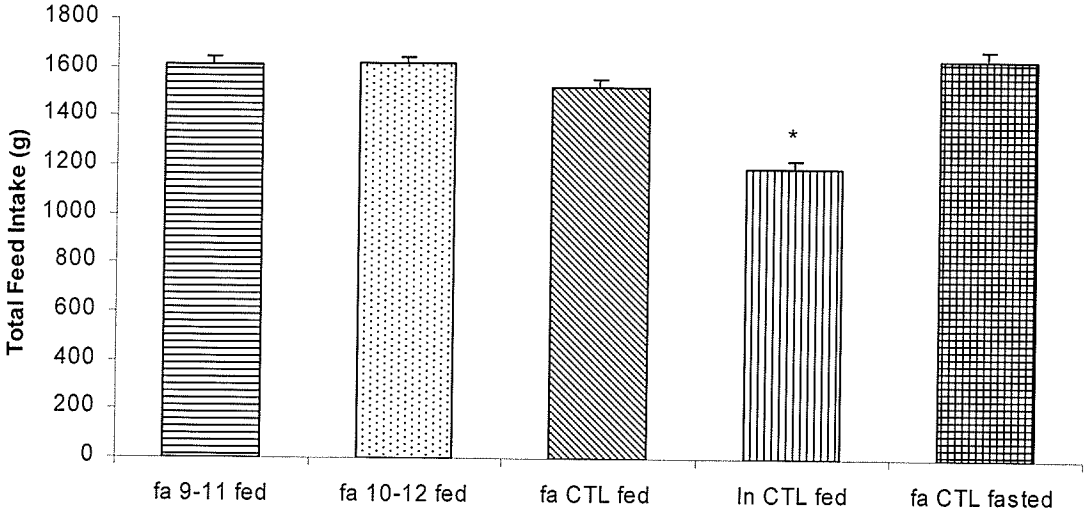


Figure 27. Total feed intake of lean and *fa/fa* Zucker rats, in the fed or fasted state, fed CLA isomers for 8 weeks. The * denotes a significant difference from fa 9-11 fed, fa 10-12 fed, fa CTL fed, and fa CTL fasted ($p<0.0001$). Data are presented as means ± SEM (n=6 or 7 per group).

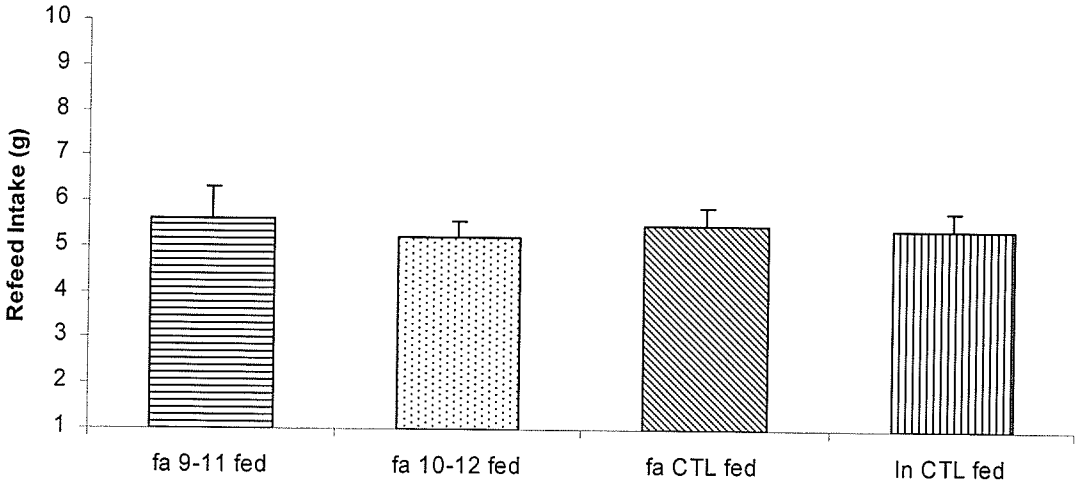


Figure 28. Re-feed intakes of lean and *fa/fa* Zucker rats fasted for 12 hours and re-fed for 2 hours. There were no significant differences among groups for re-feed intakes. Data are presented as means ± SEM (n=6 or 7).

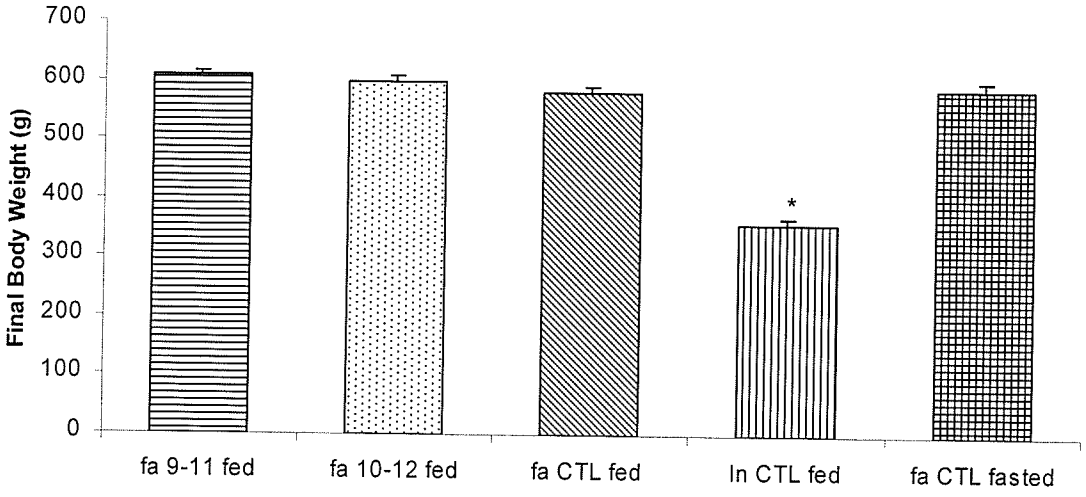


Figure 29. Final body weights of lean and *fa/fa* Zucker rats, in the fed or fasted state, fed CLA isomers for 8 weeks. The * denotes a significant difference from fa 9-11 fed, fa 10-12 fed, fa CTL fed, and fa CTL fasted ($p<0.0001$). Data are presented as means ± SEM (n=6 or 7).

Absolute and Adjusted Liver Weights

Similar to Study 1, there was a genotype difference in absolute liver weights such that the ln CTL fed rats had significantly lower absolute liver weights compared to rats in the fa CTL fed group. Although the absolute liver weight of the fa CTL fed group was slightly higher than that of the fa CTL fasted group, this difference was not statistically significant. The fa 10-12 fed group had a significantly lower absolute liver weight compared to the fa CTL fed group; however, the absolute liver weight of the fa 9-11 fed group was not different from the fa CTL fed group. There was no significant difference in absolute liver weight between the fa 9-11 fed and fa 10-12 fed groups (Figure 30).

When examining liver weight adjusted for body weight, there were no differences between the fa CTL fasted and fa CTL fed groups, nor between the fa CTL fed and ln CTL fed groups (Figure 31). Adjusted liver weights of the fa 9-11 fed and fa 10-12 fed groups were both significantly reduced compared to the fa CTL fed group.

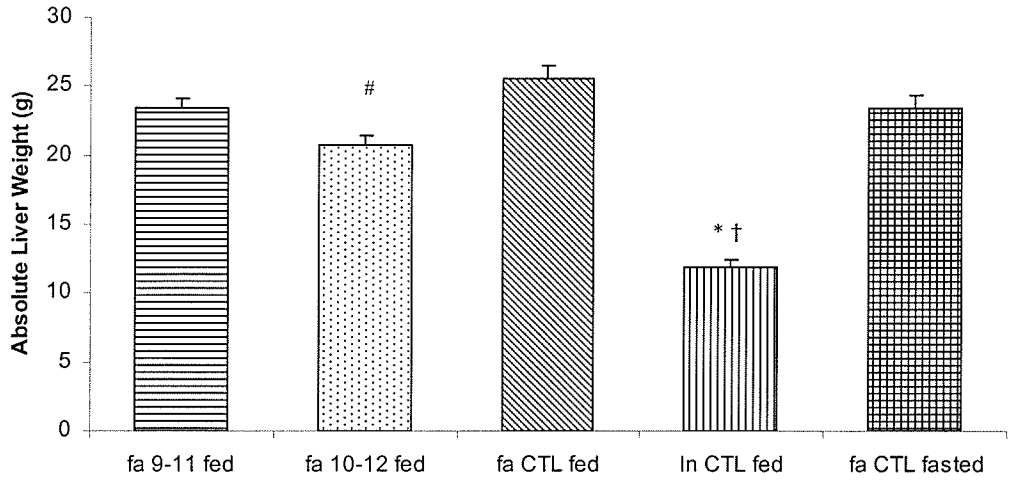


Figure 30. Absolute liver weights of lean and *fa/fa* Zucker rats, in the fed or fasted state, fed CLA isomers for 8 weeks. There was a significant group effect ($p<0.0001$). The # denotes a significant difference from fa CTL fed ($p<0.01$). The * denotes a significant difference from fa CTL fed ($p<0.001$). The † denotes a significant difference from fa CTL fasted ($p<0.001$). Data are presented as means ± SEM (n=6 or 7 per group).

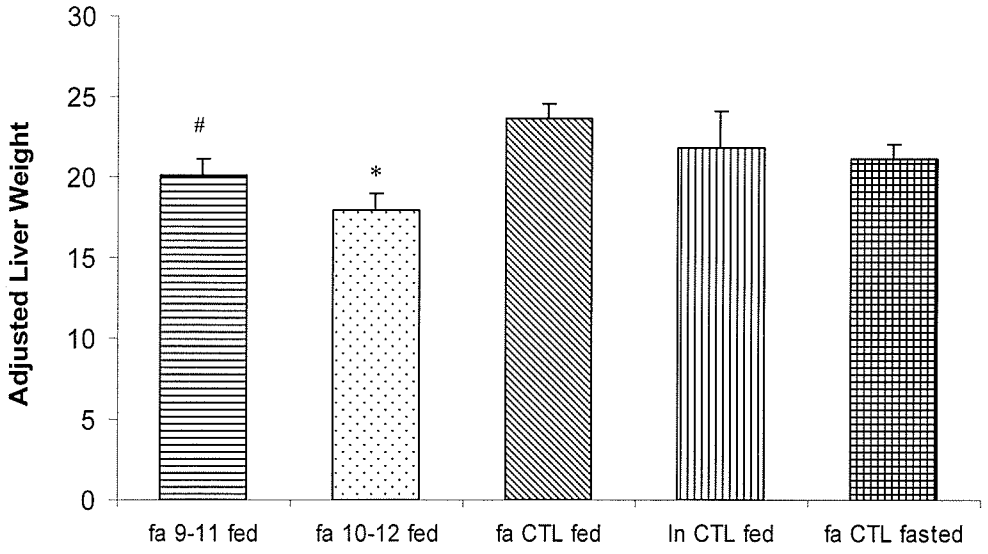


Figure 31. Adjusted liver weights of lean and *fa/fa* Zucker rats, in the fed or fasted state, fed CLA isomers for 8 weeks. There was a significant group effect ($p<0.001$). The # denotes a significant difference from fa CTL fed ($p<0.01$). The * denotes a significant difference from fa fed CTL ($p<0.001$). Data are presented as adjusted means ± SEM (n=6 or 7 per group), where liver weight was adjusted for body weight using ANCOVA.

Hepatic Lipid Concentration

Hepatic lipid concentration was 65% lower in the ln CTL fed group compared to the fa CTL fed group. Hepatic lipid concentration was not different between the fa CTL fed and the fa CTL fasted groups, nor between the fa CTL fed and the fa 9-11 fed groups. However, hepatic lipid concentration of the fa 10-12 fed group was 54.0% lower than the fa CTL fed group, and 48.0% lower than the fa 9-11 fed group (Figure 32).

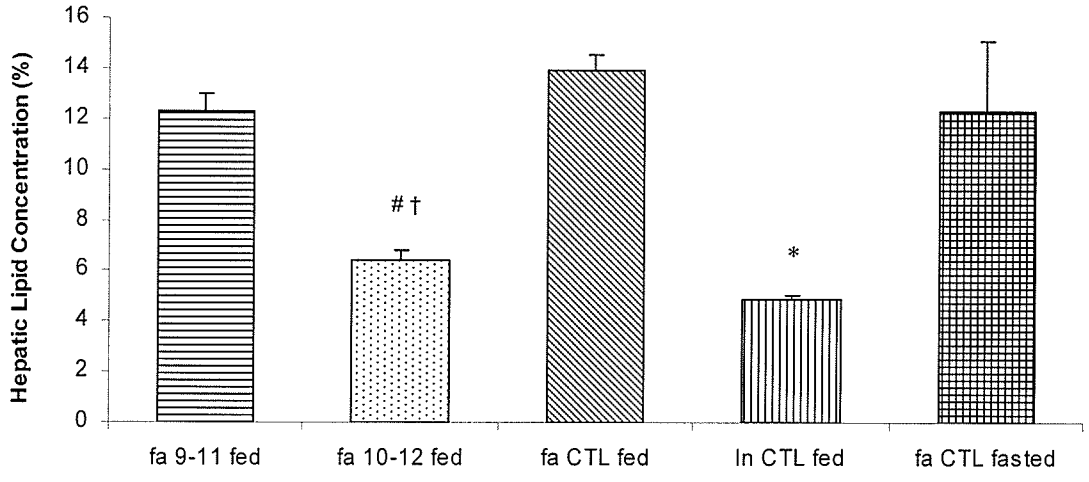


Figure 32. Hepatic lipid concentration of lean and *fa/fa* Zucker rats, in the fasted or fed state, fed CLA isomers for 8 weeks. There was a significant group effect ($p<0.0005$). The * denotes a significant difference from fa CTL fed ($p<0.01$). The # denotes a significant difference from fa CTL fed ($p=0.001$). The † denotes a significant difference from fa 9-11 fed ($p<0.05$). Data are presented as means ± SEM (n=4 per group).

Hepatic Proteins

i. AMPK

Based on results from ANOVA, there was a trend (p=0.081) towards a significant difference in hepatic AMPK protein levels. There was no significant difference in AMPK protein based on genotype (In CTL fed vs. fa CTL fed) or nutritional state (fa CTL fed vs. fa CTL fasted). There was a trend for AMPK protein levels to be lower in the fa 10-12 fed group compared to the fa CTL fed group (p=0.0575) and the fa 9-11 fed group (p=0.0635) (Figure 33).

ii. pAMPKα

There were no significant differences among the groups in regards to protein levels of pAMPKα (Figure 34).

iii. SREBP-1 p125

A significant effect of group was found for hepatic SREBP-1 p125 protein levels (Figure 35). When examining differences between specific groups, there was a trend for higher SREBP-1 p125 in fa 9-11 fed compared to fa CTL fed (p=0.075). Differences among the other groups were not significant.

iv. SREBP-1 p68

There were no differences among the groups in regards to hepatic protein levels of SREBP-1 p68 (Figure 36).

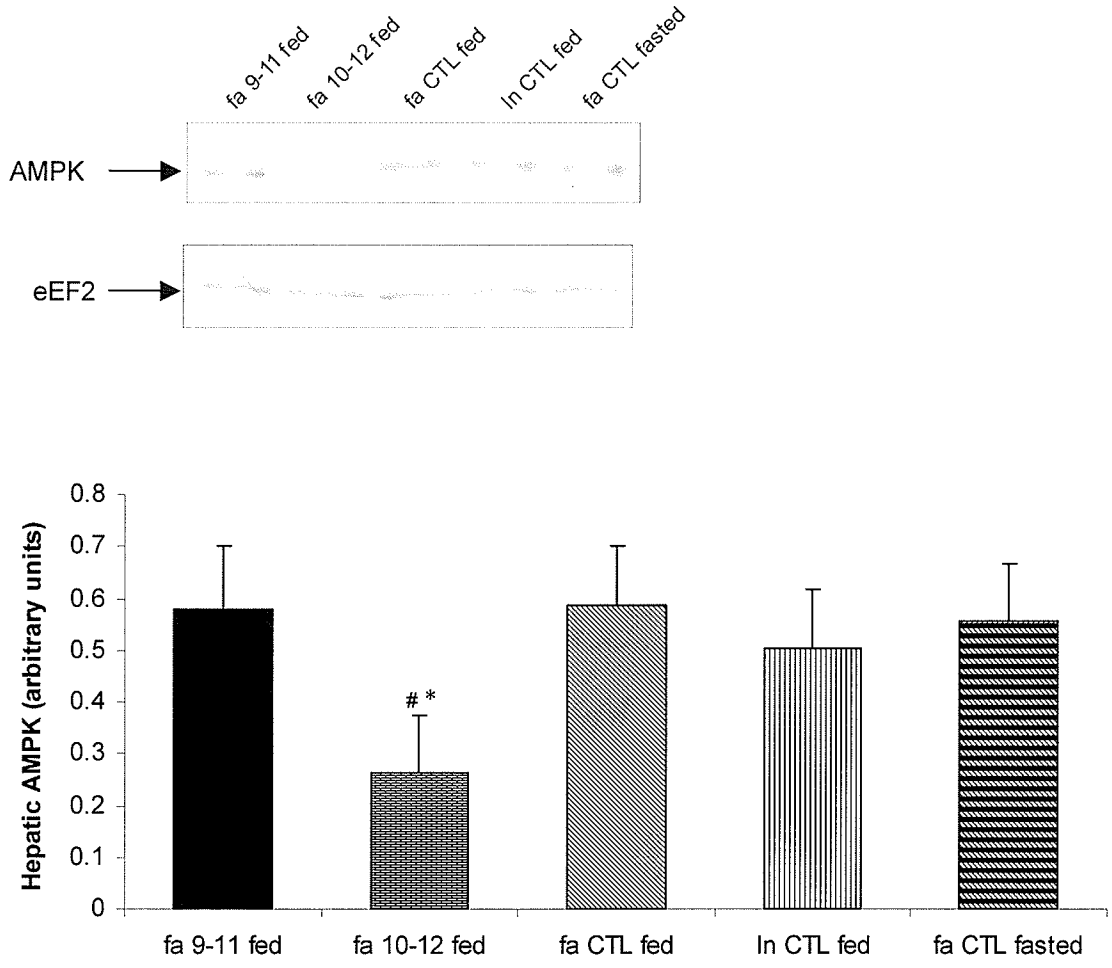


Figure 33. Hepatic AMPK protein levels in lean and *fa/fa* Zucker rats, in the fed or fasted state, fed CLA isomers for 8 weeks. There was a trend towards a group effect (p=0.081). The * denotes a trend towards being lower than fa CTL fed (p=0.0575). The # denotes a trend towards being lower than fa 9-11 fed (p=0.0635). Data are presented as adjusted means ± SEM (n=4 per group), where AMPK values were adjusted for intensity and loading controls using ANCOVA.

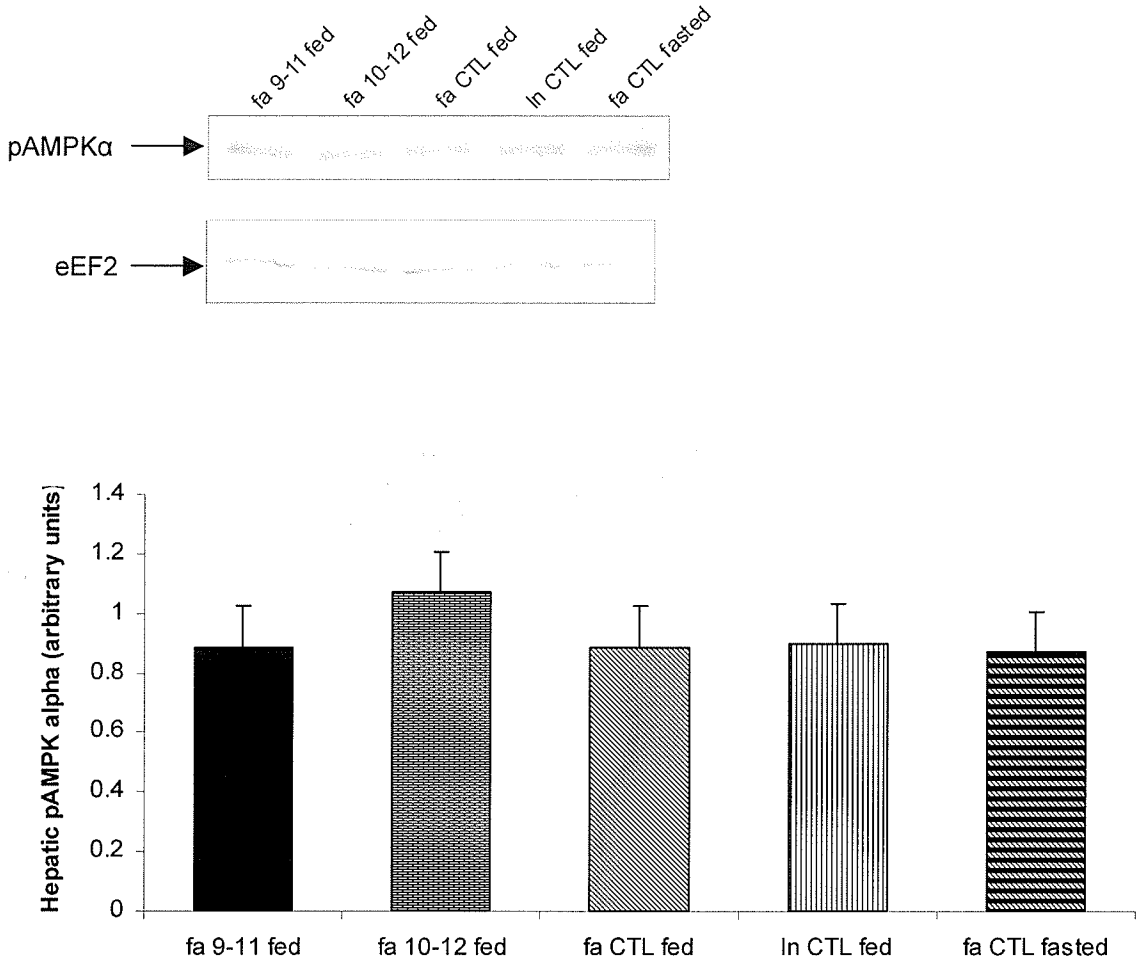


Figure 34. Hepatic pAMPKα protein levels in lean and *fa/fa* Zucker rats, in the fed or fasted state, fed CLA isomers for 8 weeks. There was no effect of group. Data are presented as adjusted means ± SEM (n=4 per group), where values for pAMPKα wereadjusted for intensity and loading control and total hepatic AMPK using ANCOVA.

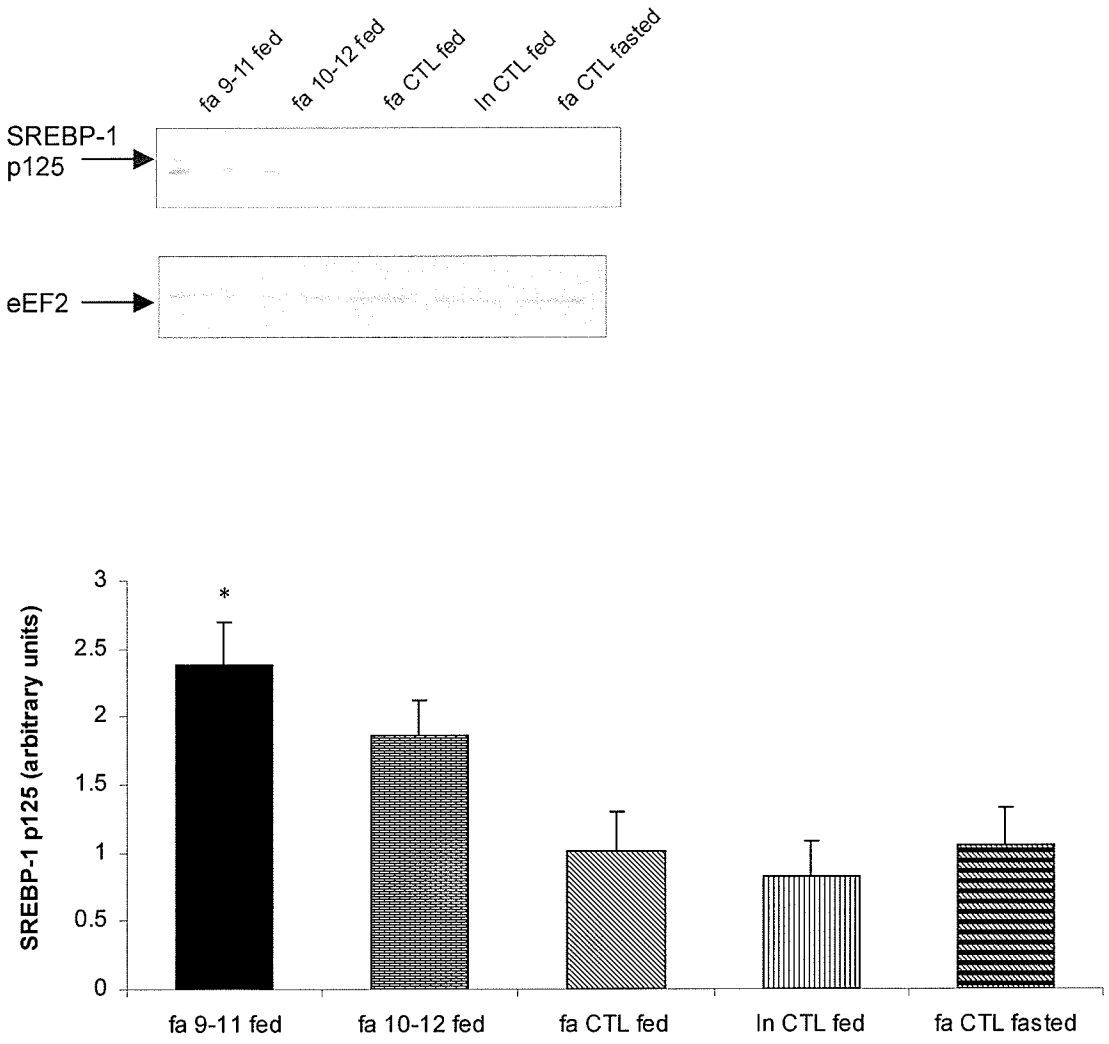


Figure 35. Hepatic SREBP-1 p125 protein levels in lean and *fa/fa* Zucker rats, in the fed or fasted state, fed CLA isomers for 8 weeks. There was a significant effect of group (p=0.0179). The * denotes a trend towards being higher than fa CTL fed (p=0.075). Data are presented as adjusted means ± SEM (n=4 per group), where values for SREBP-1 p125 were adjusted for intensity and loading control using ANCOVA.

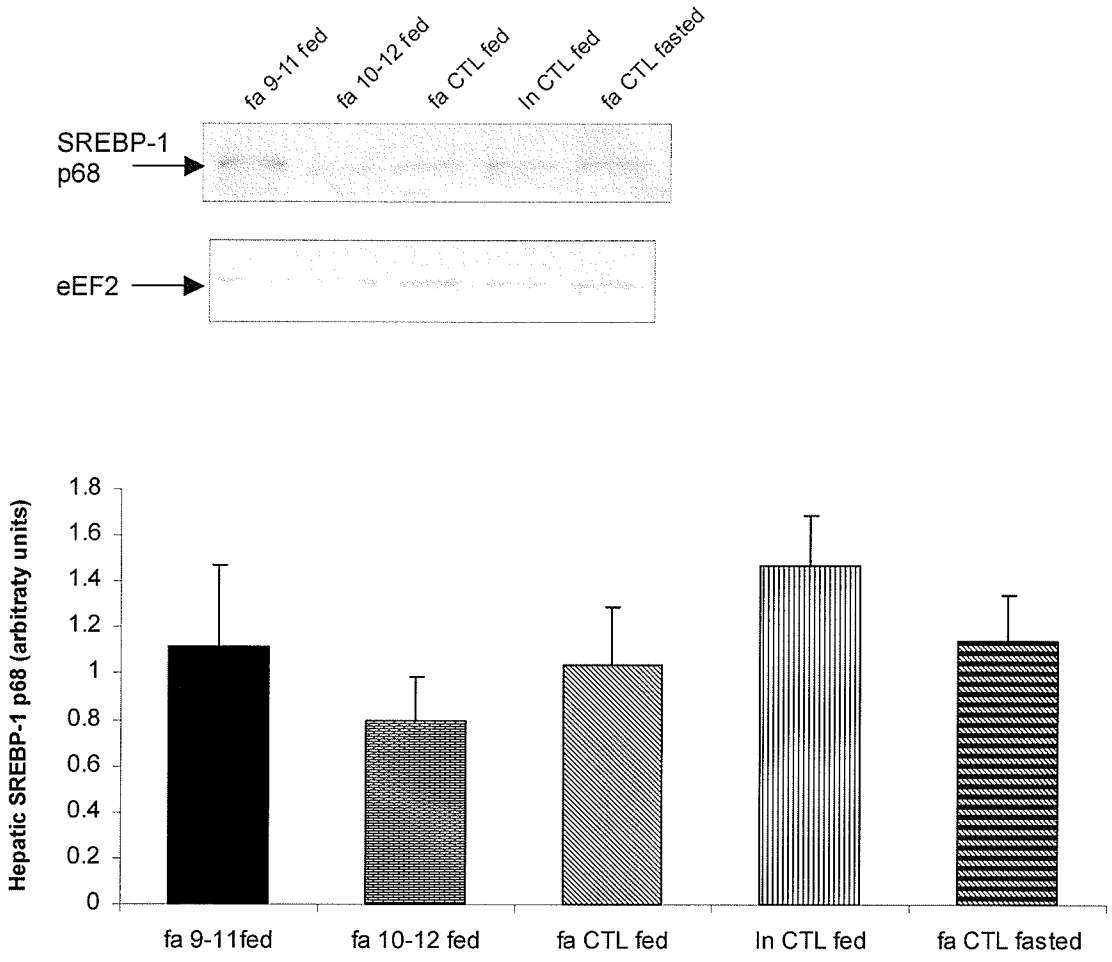


Figure 36. Hepatic SREBP-1 p68 protein levels in lean and *fa/fa* Zucker rats, in the fed or fasted state, fed CLA isomers for 8 weeks. There was no effect of group. Data are presented as adjusted means ± SEM (n=4 per group), where SREBP-1 p68 values were adjusted for intensity and loading controls and total hepatic SREBP-1 using ANCOVA.

DISCUSSION

Baseline Analysis

Baseline and endpoint rats were 6 weeks and 14 weeks of age, respectively, at termination. By 6 weeks of age, *fa/fa* Zucker rats were already insulin resistant, as evidenced by higher serum insulin concentrations compared to lean Zucker rats of the same age (Figure 7). In fact, fasting serum insulin concentrations of 6-week-old *fa/fa* Zucker rats were already significantly higher than 14-week-old lean Zucker rats. Similarly, serum C-peptide was already higher in *fa/fa* Zucker rats at 6-weeks of age (Figure 8). Elevated liver weights and hepatic steatosis were already present at 6 weeks of age in *fa/fa* Zucker rats. In the current study, hepatic lipid concentration was 51% higher in the *fa/fa* Zucker baseline rats compared to the lean baseline rats (Figure 9).

One of the most interesting findings from the baseline vs. endpoint analysis was the result of serum TAG and FFA analyses. By 6 weeks of age, *fa/fa* Zucker rats had significantly higher serum TAG and FFA compared to lean Zucker rats of the same age; furthermore, the degree of hypertriacylglycerolemia and elevated FFA was similar to that observed in the 14-week-old *fa/fa* Zucker rats (Figures 10 and 11).

All of these results suggest that at 6 weeks of age, *fa/fa* Zucker rats are hyperinsulinemic, hyperlipidemic, and have hepatic steatosis. These results are consistent with what has been previously found in young *fa/fa* Zucker rats.

Coimbra et al. (2000) found that 6-week-old male *fa/fa* Zucker rats were hypertriacylglerolemic and hyperinsulinemic compared to lean littermates. According to Kreif and Bazin (1991), hypertriacylglycerolemia can be detected as early as 2 weeks of age, and hyperinsulinemia and hepatic steatosis can be detected as early as 3 weeks of age in the *fa/fa* Zucker rat.

Liver dysfunction was present in our 6-week-old *fa/fa* Zucker rats, as evidenced by significantly higher serum ALT concentration compared to 6-week-old lean rats (Figure 13). According to results from the current study, impaired liver function in the 6-week-old *fa/fa* Zucker rats continues to decline, as serum ALT concentrations in 14-week-old *fa/fa* rats is significantly higher than that of the 6-week-old *fa/fa* rats. To the author's knowledge, this is the first study that has assessed liver function in young *fa/fa* Zucker rats.

Dietary CLA Analysis

Total Feed Intake and Final Body Weight

As expected, total feed intake was significantly higher in the *fa/fa* control rats than the lean control rats (Figures 14 and 15). The 38% higher consumption of feed observed in the *fa/fa* rats in this study is due to the lack of functional leptin receptors in these animals. Although not affected by dietary CLA in the lean rats, total feed intake of *fa/fa* rats fed the 10-12, BIO, and NCK diets was reduced by 16%, 9%, and 12%, respectively. Reductions were not found with the 9-11 or TOG diets, suggesting that the c9,t11 isomer has no effect on feed

intake in *fa/fa* Zucker rats. The inability of the TOG diet to produce the same effect on feed intake, despite its t10, c12 content, is inexplicable at this point.

At 14-weeks of age, *fa/fa* rats weighed significantly more than lean rats. Body weight was not changed with dietary CLA in lean rats and, despite reduced feed intake in the fa 10-12, fa BIO, and fa NCK groups, was not changed in the *fa/fa* rats. These results are consistent with work done by Sisk et al. (2001), who found that 35 days of feeding a 0.5% CLA mixture to growing male lean and *fa/fa* Zucker rats did not lower body weight in either genotype. The maintenance of body weight in spite of reduced feed intake suggests that t10,c12 CLA may either increase fat mass or lean body mass in the *fa/fa* Zucker rat. Indeed, Diakiw (2005) reported higher visceral fat pad weights in *fa/fa* rats fed t10,c12 CLA for 8 weeks.

Ryder et al. (2001) observed reduced feed intake coupled with lower body weights and lower epididymal fat pad weights in male ZDF rats fed a mixture containing equal amounts of c9,t11 and t10,c12 CLA (50:50) at 1.5% of the diet for 2 weeks. In this study, the c9,t11 isomer alone had no effect on feed intake or body weight, which is consistent with the beneficial effect of t10,c12 seen in the current study. However, Houseknecht et al. (1998) found that feeding a 1.5% CLA mixture did not affect feed intake or final body weight in 6-week-old male ZDF rats fed treatment diets for 2 weeks. Unfortunately, the isomeric composition of the CLA mixture used in this study was not provided; therefore, it is possible that this mixture contained predominantly c9,t11 CLA, which would

account for the lack of effect on feed intake and body weight observed in this study.

In other rat models of obesity, CLA does not appear to modulate feed intake. In 5-week-old male Otsuka Long-Evans Tokushima fatty (OLETF) rats, a 1.0% CLA mixture containing equal amounts of the c9,t11 and t10,c12 isomers did not result in lower feed intake in these rats over a 4-week feeding period (Rahman et al., 2001). Although t10,c12 CLA reduced feed intake of the *fa/fa* rats in the current study, 1% dietary t10,c12 CLA for 2 weeks did not have the same effect in 6-week-old male OLETF rats (Wang et al., 2006). The lack of effect in OLETF rats could result from the relatively short feeding period; however, 2 weeks of CLA feeding in ZDF rats was sufficient to elicit reduced feed intake (Ryder et al., 2001).

The difference in response of feed intake to dietary CLA in Zucker rats vs. OLETF rats may be due to the underlying causes of hyperphagia in these rat models. OLETF rats develop hyperphagia due to a lack of functional cholecystokinin receptors, while the cause of hyperphagia in *fa/fa* Zucker rats and ZDF rat is the dysfunctional leptin receptor. Leptin has an inhibitory effect on neuropeptide Y, a neurotransmitter involved in regulation of appetite. Neuropeptide Y gene expression is elevated in the brain of *fa/fa* Zucker rats (Bogaka et al., 2004). Thus, the ability of CLA to reduce feed intake in the *fa/fa* Zucker rat may due to either an improvement in leptin binding and signaling, or by bypassing the leptin receptor and directly modulating the proteins that regulate neuropeptide Y expression. These theories have yet to be tested.

Absolute and Adjusted Liver Weights

In both Study 1 and Study 2, lean control rats had lower absolute liver weights than *fa/fa* control rats (9.54 g for ln CTL vs. 24.5 g for fa CTL in Study 1; 11.9 g for ln fed CTL vs. 25.6 g for fa fed CTL in Study 2). Similarly, adjusted liver weights were lower in the lean control rats compared to the *fa/fa* control rats in both studies; however, the magnitude of change was much higher when animals were in the fasted state compared to the fed state (34% lower in Study 1, while only 7.6% lower in Study 2). According to results of Study 1, dietary CLA had no effect on absolute and adjusted liver weights in the lean genotype (Figures 16 and 17). This is consistent with findings from Yamasaki et al. (2003), who found no change in liver weight after 3 weeks of CLA-feeding in Sprague Dawley rats.

In the current study, feeding the 10-12, TOG, BIO, or NCK diets all resulted in a significant reduction in absolute and adjusted liver weights in the *fa/fa* genotype (28.2%-29.4% reduction in absolute liver weight; 29.6%-36.4% reduction in adjusted liver weight), suggesting that t10,c12 CLA produces this effect on liver weight. In a previous study conducted in our laboratory, feeding a mixture of CLA isomers at 1.5% (wt/wt) of the diet reduced liver weight relative to body weight in male *fa/fa* Zucker rats by 26% (Noto et al., 2006). A possible explanation for the difference between the reductions in adjusted liver weights in the current study compared to relative liver weights in Noto et al. (2006) is the method of calculating relative liver weight. In the current study, we used covariate statistics to adjust liver weight for body weight, whereas Noto et al.

report relative liver weight as g/100 g body weight. Using this method of dividing liver weight by body weight assumes body weight is a control variable. This assumption is only appropriate if there is a direct relationship between the two variables (i.e. as liver weight increase, body weight increases; if liver weight decreases, body weight increases). However, this was not the case in our study: dietary t10,c12 CLA reduced liver weight in *fa/fa* rats, but did not reduce body weight (Figures 15 and 16). Therefore, we used covariate statistics to adjust liver weight for body weight.

Similar to Noto et al. (2006), Nagao et al. (2006) also found a 26% reduction in relative liver weight in male *fa/fa* Zucker rats fed CLA isomers supplemented at 1% (wt/wt) of the diet for 8 weeks. In this study, relative liver weight was expressed in the same manner as by Noto et al. (2006). Conversely, Wang et al. (2006) observed no change in relative liver weight in OLETF rats fed a diet supplemented with 1% (wt/wt) c9,t11 or t10,c12 CLA. This feeding trial only lasted for 2 weeks; therefore, it is possible that a longer feeding period is required to observe reductions in liver weight induced by CLA feeding.

Hepatic Lipid Concentration

At the end of both the current studies, hepatic lipid concentration in the *fa/fa* control rats was significantly higher than the lean control rats (71.3% lower in Study 1 and 65% lower in Study 2). Dietary CLA treatment did not affect hepatic lipid concentration in lean Zucker rats. In the *fa/fa* genotype, all diets containing the t10,c12 isomer (10-12, TOG, BIO, and NCK) produced a marked

reduction in hepatic steatosis (average reduction of 61.3%); furthermore, hepatic lipid concentrations were restored almost to the level found in the lean rats (Figures 18 and 32). Incredibly, dietary t10,c12 CLA was able to reduce hepatic lipid concentration to levels lower than that observed in 6-week-old *fa/fa* baseline rats. Feeding the c9,t11 isomer did not elicit a lipid-lowering effect in the liver of *fa/fa* rats. The magnitude of reduction in hepatic lipid concentration is consistent with what was found previously in our laboratory, where a CLA mixture supplemented at 1.5% (wt/wt) of the diet reduced hepatic steatosis by 62% in male *fa/fa* Zucker rats (Noto et al., 2006). Similarly, Nagao et al. (2005) observed a 78% reduction in hepatic lipid concentration after feeding male *fa/fa* Zucker rats a 1% (wt/wt) CLA mixture for 8 weeks. In their study, hepatic lipid concentration was measured using an enzymatic colourimetric assay to determine the TAG concentration of hepatic lipid extracts, which could explain the higher reduction seen in the study by Nagao et al. (2006) when compared with studies conducted in our laboratory. Colourimetric determination of hepatic lipid concentration is most likely more accurate than the gravimetric method we used, as the gravimetric method involves transferring solvents to and from various containers, and also relies on visual determination of quantities.

Short-term CLA feeding has also been shown to reduce hepatic steatosis. When growing female *fa/fa* Zucker rats were given 1.5 g/kg body weight of t10,c12 CLA via intragastric gavage for only 21 days, hepatic lipid concentration was reduced by 33%, compared to rats given a corn oil vehicle, c9,t11 CLA alone or the c9,t11 and t10,c12 isomers in combination (Sanders et al., 2004). In 5-

week-old OLETF rats fed 9% (wt/wt) safflower oil plus 1% (wt/wt) t10,c12 CLA, hepatic TAG concentration was reduced in as little as 10 days (Nagao et al., 2003). Results from the previous two studies, combined with our results, support the hypothesis that the t10,c12 CLA isomer is responsible for reducing hepatic steatosis in rats. In contrast, the actions of the t10,c12 isomer on hepatic lipid concentration in mice are opposite to rats, as this isomer has been shown to induce hepatic steatosis in mice (Clément et al., 2002).

In the current study, reduced hepatic lipid concentrations correlated well with reduced serum ALT concentrations, a marker of liver dysfunction. Associations between hepatic lipid content, as assessed by ^{1}H-magentic resonance spectroscopy, and serum ALT concentrations have been previously reported in humans (Westerbacka et al., 2004; Schindhelm et al., 2006).

Lipidemia

i. Cholesterol

At the end of the 8-week feeding period of Study 1, fasting serum total cholesterol was significantly higher in the *fa/fa* control rats compared to the lean control rats (24.7 ± 6.2 mmol/L vs. 11.2 ± 2.3 mmol/L, respectively). CLA-feeding had no effect on serum total cholesterol concentrations in the lean genotype. Kim et al. (2005) found that feeding a 1.5% (wt/wt) CLA mixture supplemented into a vitamin E-deficient diet to male Sprague Dawley rats (a lean rat model) resulted in significantly lower plasma total cholesterol concentrations after 5 weeks. However, when Kloss et al. (2005) fed 6-week-old male Sprague Dawley

rats corn oil plus a 1.5% (wt/wt) CLA mixture for 28 days, they did not find a reduction in serum total cholesterol concentrations. It is possible that differences in the background diet or major lipid source of the diet, or even differences in breed, are responsible for the variation in the ability of CLA to reduce serum total cholesterol concentrations in lean rats.

In the present study, all diets containing the t10,c12 CLA isomer significantly reduced serum total cholesterol concentrations, on average, by 26.3% in the *fa/fa* rats, while the c9,t11 isomer had no effect on this parameter (Figure 19). There is limited data on dietary CLA and serum total cholesterol in obese rats, possibly because other animal models are more reflective of cholesterol metabolism in humans. However, a few studies do exist. In the male *fa/fa* Zucker rat, a dietary CLA mixture has previously been shown to reduce serum total cholesterol by 17.2% (Nagao et al., 2005) and 37% (Noto et al., 2006). In male OLETF rats, reduced serum cholesterol concentrations were observed with 1% (wt/wt) t10,c12 CLA supplemented into 9% (wt/wt) safflower oil in as little as 2 weeks; however, this difference was not statistically significant (Nagao et al., 2003). Perhaps with a larger sample size or a longer feeding period this difference would have reached statistical significance. Feeding with the c9,t11 isomer in this study did not affect serum cholesterol. Coupled with the results from the current study, it appears that t10,c12 CLA is the isomer that reduces serum total cholesterol in rats.

The cholesterol-lowering effect of CLA has also been observed in other species. In male Syrian Golden Hamsters fed a hypercholesterolemic diet for 12

weeks, 0.5% (wt/wt) of either c9,t11 CLA or t10,c12 CLA significantly reduced plasma total cholesterol (Wilson et al., 2006). Unfortunately, HDL as well as non-HDL cholesterol was affected. However, when male C57BL/6J mice were fed 0.6% (wt/wt) CLA supplemented into a hypercholesterolemic diet, serum total cholesterol was reduced by 25%, and the HDL:total cholesterol ratio was significantly higher in the CLA-fed mice (Lee et al., 2005). Although positive, results from studies such as these are hard to compare with results from the current study, as the cholesterol-lowering ability of CLA in conjunction with a hypercholesterolemic diet might be different from that observed in conjunction with a lipid-balanced diet.

ii. TAG

Previous analysis of rats from Study 1 revealed that the *fa/fa* rats displayed significantly higher serum TAG concentrations than the lean rats (4.88 ± 0.30 mmol/L vs. 0.576 ± 0.026 mmol/L, respectively) (Diakiw, 2005). Eight-weeks of CLA treatment did not affect serum TAG concentrations in either genotype. Although *fa/fa* rats fed the 9-11 and TOG diets had lower serum TAG concentrations compared to all other dietary groups in the *fa/fa* genotype, these differences were not statistically significant (Table 12). Large variability in serum TAG concentrations of *fa/fa* rats within the same dietary treatment group could account for the lack of significant differences in the current study.

However, other studies in Zucker rats also have found no effect of dietary CLA on serum TAG. Noto et al. (2006) observed higher serum TAG

concentrations in *fa/fa* vs. lean Zucker rats, but no changes in serum TAG after 8 weeks of feeding a 1.5% CLA mixture. In the current study, serum TAG concentrations were lower than those observed by Noto et al. (2006), where serum TAG was 3.90 ± 0.31 mmol/L in lean rats and 16.6 ± 0.94 mmol/L in *fa/fa* rats. This suggests that serum TAG in *fa/fa* Zucker rats varies based on litter or breeding colony. In yet another study using *fa/fa* Zucker rats, 5 weeks of feeding 8-week-old males and 8 weeks of feeding 6-week-old females a diet supplemented with a 0.5% CLA mixture did not result in any changes in serum TAG concentrations (Sisk et al., 2001).

The ZDF rat shares many of the same phenotypic characteristics of the *fa/fa* Zucker rat with the exception that the ZDF rat displays a very high level of hyperglycemia, and is therefore a more appropriate model for Type 2 diabetes (Peterson et al., 1990). When 7-week-old male ZDF rats were fed a diet supplemented with 1.5% (wt/wt) c9,t11 CLA or a 1.5% (wt/wt) mixture of equal amounts of the c9,t11 and t10,c12 isomers for 2 weeks, serum TAG was significantly reduced by 20.3% and 29.5%, respectively, compared to control ZDF rats (Ryder et al., 2001). Although the magnitude of reduction was higher in the group fed the mixture, this difference was not statistically significant.

Dietary CLA has been shown to reduce serum TAG concentrations in other rat models of obesity and insulin resistance. Four weeks of feeding 5.5% (wt/wt) safflower oil supplemented with a 1.0% (wt/wt) CLA mixture containing equal amounts of c9,t11 and t10,c12 CLA, delivered in the FFA form, reduced serum TAG concentrations by 31.2% in OLETF rats (Rahman et al., 2001).

Interestingly, serum TAG concentrations in OLETF rats given the same amount of CLA, but in the TAG form, were not different than control OLETF rats. In the same rat model, Nagao et al. (2003b) found that feeding 9% safflower oil supplemented with a 1% CLA mixture for 4 weeks also resulted in significantly lower serum TAG concentrations when compared to control rats fed 10% safflower oil (approximately 19% lower). The same study compared serum TAG of 8-week-old OLETF rats fed the c9,t11 isomer or the t10,c12 isomer for 10 days, and found that serum TAG was approximately 24% lower in the t10,c12 group compared to the c9,t11 group. Although an ability of CLA to lower serum TAG was not found our study, results from Rahman et al. (2001) and Nagao et al. (2003b) suggest that CLA does lower serum TAG in OLETF rats, and furthermore, the t10,c12 isomer produces this lowering effect.

ii. FFA

Previous analysis of rats from Study 1 found significantly higher fasting serum FFA in *fa/fa* Zucker rats compared to lean Zucker rats after the 8-week study (1.27 ± 0.04 mmol/L in *fa/fa* Zucker rats vs. 0.520 ± 0.015 mmol/L in lean Zucker rats) (Diakiw, 2005). None of the CLA-containing diets had any effect on serum FFA in either the lean or *fa/fa* genotypes (Table 12).

This absence of a FFA-lowering effect of CLA is in contrast to what has been previously found in the *fa/fa* Zucker rat and other rat models of obesity and insulin resistance. In 8-9 week-old female *fa/fa* Zucker rats, 0.3 g/kg body weight and 1.5 g/kg body weight of a 50:50 mixture of c9,t11 and t10,c12 CLA for 20

days reduced serum FFA by 15.2% and 16.5%, respectively, compared to rats given the same amount of corn oil (Teachey et al., 2003). Although CLA treatment did lower serum FFA concentrations in this study, no dose-response was observed. Similarly, 8-week-old male ZDF rats given 5% (wt/wt) corn oil supplemented with a 1.5% (wt/wt) CLA mixture for 2 weeks had significantly lower serum FFA concentrations than both control-fed ZDF rats and control-fed lean Zucker rats (Houseknecht et al., 1998). The extent of FFA-lowering was similar to that seen with administration of 0.2% troglitazone, a member of the thiazoledinedione family of anti-diabetic drugs (Houseknecht et al., 1998). When Rahman et al. (2001) fed 5-week-old male OLETF rats 5.5% (wt/wt) safflower oil plus a 1.0% (wt/wt) CLA mixture containing equal amounts of the c9,t11 and t10,c12 isomers, as FFA, serum FFA concentrations were reduced by 32.2% compared to a group fed 6.5% (wt/wt) safflower oil. This reduction returned serum FFA concentrations to those observed in lean Long-Evans Tokushima Otsuka rats. Interestingly, the same amount of CLA administered in the TAG form had no effect on serum FFA in this study.

Previous research has suggested that the t10,c12 isomer is responsible for reducing serum FFA concentrations in rats. For example, Henriksen et al. (2003) examined the effects of CLA in 8-9 week old female *fa/fa* Zucker rats and found isomer-specific effects. When rats were given 1.5 g/kg body weight of c9,t11 CLA for 21 days, no effect on serum FFA was observed; however, when given the same dose of t10,c12 CLA or a mixture of the c9,t11 and t10,c12 isomers together for the same duration, both treatments resulted in significantly

lower plasma FFA concentrations when compared to treatment with corn oil. Similarly, Ryder et al. (2001) observed a 24.6% reduction in serum FFA concentrations after 2 weeks of feeding 7-week-old male ZDF rats a 1.5% CLA mixture (50:50 composition of c9,t11 and t10,c12 CLA). Feeding with the same amount of the c9,t11 isomer alone for the same duration did not result in lower serum FFA concentrations in ZDF rats.

Serum Adipocytokines

i. Leptin

Previous analysis of rats from Study 1 revealed that, as expected, the *fa/fa* CTL rats had significantly higher serum leptin concentrations than the lean CTL rats. Dietary CLA had no effect on serum leptin concentrations in the lean rats; however, significant reductions were observed with dietary t10,c12 CLA, with an average reduction in the 10-12, TOG, BIO, and NCK groups of 15% (Table 11). Dietary c9,t11 CLA did not reduce hyperleptinemia in *fa/fa* rats.

Numerous studies have shown that dietary CLA attenuates serum leptin concentrations in animal models of obesity. Like *fa/fa* Zucker rats, OLETF rats are hyperleptinemic, although the cause of hyperleptinemia is slightly different between the two rat models (Wang et al., 2006). Lack of functional CCK receptors cause the OLETF rat to become hyperphagic, resulting in increased adipose mass and higher levels of leptin mRNA.

Results from the current study, coupled with results from previous studies, agree with the hypothesis that t10,c12 CLA reduces hyperleptinemia in rats.

When Ryder et al. (2001) fed 7-week-old male ZDF rats 1.5% (wt/wt) of the c9,t11 isomer alone, or 1.5% (wt/wt) of a 50:50 mixture of c9,t11 and t10,c12 CLA, serum leptin concentrations were reduced by 30% in the rats fed the 50:50 CLA mixture, while the c9,t11 isomer alone had no effect. Likewise, Nagao et al. (2003) and Wang et al. (2006) observed significantly lower serum leptin concentrations in growing male OLETF rats fed safflower oil supplemented with 0.5% (wt/wt) or 1% (wt/wt) t10,c12 CLA for 2 to 3 weeks. In both of these studies, leptin mRNA levels in white adipose tissue of rats fed t10,c12 CLA were reduced, suggesting that t10,c12 CLA influences gene expression of leptin.

Leptin is the 16 KDa protein product of the *ob* gene. In humans and rodents, plasma leptin concentration and mRNA levels in white adipose tissue are highly correlated with BMI (Maffei et al., 1995; Freiderich et al., 1995; Considine et al., 1996). Increased leptin expression in white adipose tissue is a major contributor to hyperleptinemia (Zhang et al., 1997). Presumably, reducing adipose mass reduces leptin mRNA level and therefore also plasma concentrations. However, the attenuation of hyperleptinemia brought on by dietary t10,c12 CLA in Study 1 was not associated with reduced adipose mass (Diakiw, 2006), suggesting that t10,c12 CLA modulates serum leptin concentration by another mechanism.

In an interesting study, Zhang et al. (1997) examined the involvement of the leptin receptor in control of leptin production. Ten-day-old Zucker rat pups were separated into 3 groups: those homozygous for the functioning leptin receptor (+/+), those homozygous for the leptin receptor mutation (*fa/fa*), and

those heterozygous for the leptin receptor mutation (*fa*/+). Despite comparable fat mass among the three groups, serum leptin concentrations were lowest in the +/+ rats and highest in the *fa/fa* rats, while fa/+ rats had intermediate serum leptin concentrations. This pattern of serum leptin was mirrored by leptin expression in white adipose tissue: compared to +/+ rats, leptin mRNA was 1.6-fold higher in the *fa/fa* rats and 26% higher in the *fa*/+ rats. Results from this study suggest that the leptin receptor is part of a negative feedback mechanism whereby leptin produced by the adipocyte enters the blood, binds to the leptin receptor on the adipocyte, which then signals to the adipocyte nucleus to shut-off leptin production. In *fa/fa* rats, the inability of the leptin receptor to sense the presence of serum leptin causes a breakdown in this negative feedback mechanism and leptin production by the adipocyte does not cease. This increases leptin secretion into the blood, further worsening hyperleptinemia.

How the leptin receptor communicates with the adipocyte nucleus to stop leptin production is unknown. Again using 10-day old *fa/fa, fa*/+ and +/+ Zucker rats, Zhang et al. (2001) found that leptin receptor feedback regulation of leptin expression in brown adipose tissue occurs primarily via central nervous system-mediated effects of leptin on sympathetic system activity, while leptin receptor control of leptin expression in white adipose tissue occurs via mechanisms not directly dependent on sympathetic nervous system activity. Whatever the signal, it plausible that attenuation of hyperleptinemia observed with dietary t10,c12 CLA occurs via up-regulation of this unknown pathway (Figure 37). This hypothesis has implications for leptin action in other tissues expressing leptin receptors,

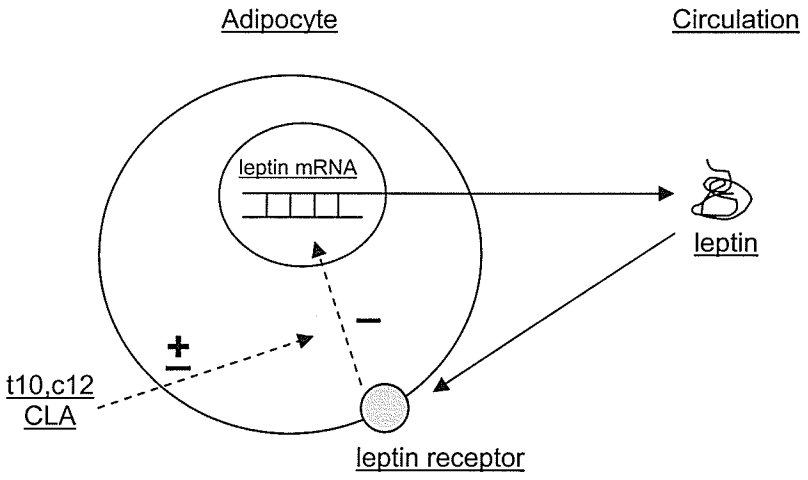


Figure 37. Hypothesis of altered leptin production by t10,c12 CLA via effects on the leptin-leptin receptor-leptin feedback loop.

including the liver. This unknown signal may also mediate, for example, hepatic lipid metabolism.

In the current study, attenuation of hyperleptinemia was associated with reduced liver lipid concentration ($r=0.62$, $p<0.0001$), suggesting that improved leptin signal transduction in the liver is implicated in reduction of hepatic steatosis. Leptin promotes fatty acid oxidation by activation of AMPK (Daval et al., 2006); however, this mechanism is likely not involved in reduced hepatic steatosis observed in our study, as we did not observe changes in AMPK activation with dietary t10,c12 CLA (Figures 21 and 31).

ii. Adiponectin

It is well-established that plasma concentrations of adiponectin are lower in obese individuals than in those of normal weight (Arita et al., 1999; Hara et al.,

2003; Silha et al., 2003). In ZDF rats, plasma concentrations of adiponectin are also significantly lower compared to lean littermates (Altomonte et al., 2003). Interestingly, previous analysis of serum from rats in Study 1 by Zirk (2005) found higher serum adiponectin concentrations in the *fa/fa* Zucker rats than the lean Zucker rats (Table 9). This unexpected result, however, is consistent with other studies in Zucker rats (Oana et al., 2005).

There was a significant effect of diet on serum adiponectin, with rats fed the 10-12, TOG, and BIO having higher serum adiponectin concentrations compared to the control groups (Table 10). Interestingly, serum adiponectin was the only parameter examined in this study in which treatment with the t10,c12 diet had an effect in both lean and *fa/fa* rats. Feeding with the c9,t11 isomer did not result in higher serum adiponectin in either genotype, suggesting that, in this model, the t10,c12 isomer is responsible for raising serum adiponectin.

Other researchers have found that CLA raises plasma adiponectin concentrations. Nagao et al. (2003b) found that feeding 7-week old male ZDF rats 5% (wt/wt) safflower oil plus a 1% (wt/wt) CLA mixture for 8 weeks resulted in almost a 2-fold increase in plasma adiponectin. In addition, Nagao et al. (2005) found that 8 weeks of feeding 7-week old male *fa/fa* Zucker rats a 1% (wt/wt) CLA mixture resulted in significantly higher hepatic levels of adiponectin compared to control rats. However, this elevation in hepatic adiponectin was not associated with higher hepatic mRNA levels of either of the two adiponectin receptors.

Adiponectin is inversely associated with hepatic lipid content, independent of obesity (Maeda et al., 2005; Yoon et al., 2005). In Study 1, however, there was no correlation between serum adiponectin and hepatic lipid concentration, suggesting that improvement in hepatic steatosis elicited by t10,c12 CLA was not related to elevated serum adiponectin and its potential effects on liver lipid metabolism. Full-length adiponectin has been reported to activate AMPK in liver (Yamauchi et al., 2002). AMPK activation increases fatty acid oxidation in skeletal muscle and liver by decreasing malonyl-CoA concentrations; however, the long-term regulation of lipid oxidation by AMPK appears to be related to its ability to activate PPAR and peroxisome proliferator activated receptor gamma coactivator-1 (Lee et al., 2006). In both the fasted and fed states, AMPK activation was not affected by dietary CLA, possibly explaining the lack of relationship between liver lipid concentration and serum adiponectin.

Plasma adiponectin is also correlated with serum TAG and FFA in normal-weight, overweight and obese individuals (Matsubara et al., 2002; Yang et al., 2002; Kazumi et al., 2004). In Study 1, serum adiponectin in rats fed t10,c12 CLA was elevated; however, this beneficial effect of dietary t10,c12 CLA was not accompanied by reductions in serum TAG or FFA concentrations.

Insulin Resistance

According to analysis by Diakiw (2005), fasting serum insulin concentrations were 16.5-fold higher in the *fa/fa* control rats than in the lean control rats from Study 1, confirming the presence of hyperinsulinemia and

insulin resistance in the *fa/fa* Zucker rats. While the euglycemic-hyperinsulinemic clamp technique is the gold standard for assessing insulin resistance, fasting insulin levels correlate well with the degree of insulin sensitivity as assessed by the clamp method, and can therefore be used as an indicator of insulin resistance (Laasko, 1993). Other methods such as the Homeostasis Model Assessment Index of insulin resistance have been used to estimate insulin resistance by using fasting serum insulin and glucose concentrations; however, these cannot reliably be used to estimate insulin resistance in rats as these models have not been validated in animals (Wallace et al., 2004).

Eight weeks of dietary CLA had no effect on fasting serum insulin concentrations in the lean dietary groups. In the *fa/fa* rats, all diets that contained the t10,c12 isomer significantly reduced fasting serum insulin, while the c9,t11 isomer had no effect on fasting insulin (Table 12). Unlike hepatic lipid concentration, dietary t10,c12 CLA was not able to restore serum insulin to concentrations observed in 6-week-old *fa/fa* baseline rats, as serum insulin remained 42% higher in the *fa/fa* rats fed diets containing t10,c12 CLA.

Previous studies using CLA mixtures have shown that CLA improves fasting serum insulin concentrations (Houseknecht et al., 1998; Nagao et al., 2003b; Noto, 2004). Isomer-specific effects on fasting serum insulin in rats have been reported previously, and are consistent with the findings from the current study. Ryder et al. (2001) indirectly discovered that t10,c12 CLA reduces serum insulin in ZDF rats. In that study, two weeks of feeding 1.5% (wt/wt) c9,t11 CLA had no effect on fasting serum insulin, while feeding 1.5% (wt/wt) of a 50:50

mixture of c9,t11 and t10,c12 CLA significantly lowered serum insulin compared to ZDF rats fed control diet. Similarly, Henriksen et al. (2003) found that feeding a 50:50 mixture of c9,t11 and t10,c12 CLA, as well as the t10,c12 isomer alone results in reduced fasting insulin in 8-9 week-old female *fa/fa* Zucker rats.

The insulin-lowering properties of CLA may not apply to all breeds of rats. Male OLETF rats, although hyperinsulinemic compared to lean controls, did not have reduced serum insulin concentrations after 4 weeks of feeding a 1.0% (wt/wt) mixture of CLA isomers (Rahman et al., 2001).

C-peptide represents a domain of the proinsulin molecule located between the sequences that will form the α- and β-chains of insulin, allowing for proper conformational folding and sulphide bridge formation before processing forms the mature insulin molecule. During processing of proinsulin, C-peptide is cleaved from the insulin molecule and is secreted from the pancreas in equimolar amounts to insulin (Marques et al., 2004). Up to 50% of insulin secreted from the pancreas is taken up by the liver in the first pass, resulting in a relatively low amount of insulin reaching peripheral tissues (Kruszynska et al., 1987). C-peptide, however, is not subject to hepatic clearance and has a longer half-life than insulin (20-30 minutes vs. 3-5 minutes for insulin), allowing it to circulate in the plasma at levels 5-times higher than insulin (Zavaroni et al., 1987). When liver function is compromised, a larger amount of insulin reaches systemic circulation and can therefore result in an overestimation of insulin secretion by

the pancreas, making serum C-peptide concentration a more accurate marker of pancreatic insulin secretion than serum insulin concentration.

Improved hepatic clearance of insulin from the blood may account for reduced serum insulin concentrations of the *fa/fa* Zucker rats fed t10,c12 CLA (Table 12). As mentioned above, impaired liver function can result in decreased insulin clearance. In this case, the molar ratio of C-peptide to insulin can be used to estimate hepatic insulin clearance (Chevenne et al., 1999). In the current study, the C-peptide to insulin ratio was 4.42 in control lean Zucker rats and 2.48 in the control *fa/fa* Zucker rats, suggesting that serum insulin clearance is impaired in the *fa/fa* Zucker rat. The average C-peptide to insulin ratio of the *fa/fa* rats fed the 10-12, TOG, BIO, and NCK diets was 3.36, and although not restored to the level of that found in lean rats, represents an improvement in hepatic insulin clearance. There was a strong positive relationship between fasting serum insulin and ALT ($r=0.79$, $p<0.0001$), but it is unknown if the improvement in fasting hyperinsulinemia is the cause or consequence of reduced ALT. Attenuation of insulin hepatic insulin resistance may restore liver function by reducing liver lipid accumulation, or improved liver function may reduce serum insulin via increased insulin clearance.

Given that serum C-peptide of *fa/fa* rats was also reduced by feeding the t10,c12 isomer, reduced insulin output by the pancreas probably also contributed to reducing hyperinsulinemia. Neuropeptide Y suppresses insulin secretion; improved leptin signaling with consequent inhibition of neuropeptide Y would result in less suppression of insulin secretion Therefore, it is not likely that

improvement in pancreatic insulin secretion observed in the current study is due to improvement in leptin signaling.

In the current study, the close relationships between reductions in fasting serum insulin concentrations and attenuation of hepatic lipid concentration ($r=0.74$, $p<0.0001$), serum ALT concentrations ($r=0.79$, $p<0.0001$), and serum haptoglobin concentrations ($r=0.79$, $p<0.0001$) suggests that improved insulin sensitivity with dietary t10,c12 CLA reduces hepatic steatosis, improves liver function, and lessens inflammation.

Glycemia and Glucose Tolerance

The *fa/fa* Zucker rat is considered a model of obesity and insulin resistance, not Type 2 diabetes, due to the fact that this model traditionally does not develop overt hyperglycemia (Kava et al., 1990). However, results from our study show that the *fa/fa* rats had slightly, but significantly, higher 5-hour fasting serum glucose concentrations compared to the lean rats (9.35 ± 0.21 mmol/L vs. 7.52 ± 0.15 mmol/L, respectively). Interestingly, fasting glucose values in lean Zucker rats in our study were noticeably higher than values reported from other studies, which average around 4 mmol/L (Houseknecht et al., 1998). The reason for this discrepancy is unknown, but could result from differences in duration of fasting. Fasting glycemia was not affected by CLA-feeding in the lean genotype. Interestingly, feeding with the t10,c12 diet appeared to slightly raise fasting

glycemia in the *fa/fa* rats, although this concentration was not statistically different than fasting glucose values in the other *fa/fa* dietary groups.

Contrary to what was found in the present study, other research in *fa/fa* Zucker rats has found that dietary CLA reduces fasting blood glucose concentrations. In 8 to 9-week-old female *fa/fa* Zucker rats, 1.5 g/kg body weight of t10,c12 CLA given via oral gavage for 21 days produced an 11% reduction in fasting plasma glucose compared to rats given corn oil for the same amount of time (Henriksen et al., 2003). The same amounts of c9,t11 CLA and a 50:50 mixture of the c9,t11 and t10,c12 isomers did not lower fasting plasma glucose. CLA also has been shown to reduce fasting blood glucose in ZDF rats. Six-week-old male ZDF rats fed a 1.5% CLA mixture for 2 weeks had significantly lower fasting blood glucose concentrations than control ZDF rats, and were comparable to blood glucose concentrations found in lean Zucker control rats or in ZDF rats given troglitazone (Houseknecht et al., 1998). In our study, fasting serum glucose concentrations of lean and *fa/fa* Zucker rats were higher than those of lean Zucker and ZDF rats in the study by Houseknecht et al. (1998), who reported fasting blood glucose concentrations of approximately 4.17 mmol/L in lean rats and approximately 5.28 mmol/L in control ZDF rats. The large difference in our fasting serum values and those of Houseknecht et al. (1998) may be due to differences in length of fasting [5-hour fast in our study compared to a 16-hour fast in Houseknecht et al. (1998)]. In addition, glucose tolerance was determined after an intraperitoneal injection of glucose in the study by Houseknecht et al. (1998), whereas we administered glucose orally. Despite

prior training, preparing the rats for the oral glucose tolerance test in the current study may have caused stress for the rats, thus raising their blood glucose concentrations prior to testing. It has been demonstrated in rats that exposure to acute stressors results in elevations in serum or plasma glucose, and that the degree of elevation is related to the intensity of stress (Armario et al., 1986; Amario et al., 1990; de Boer, et al., 1990). In addition, Houseknecht et al. (2003) used a blood glucose meter to measure whole-blood glucose concentrations, whereas we determined serum glucose concentrations using an enzymatic colourimetric assay. It has been suggested that whole-blood glucose values determined using a glucose meter are lower than that of serum or plasma glucose concentrations determined using enzymatic methods (Luo et al.,2006) All of these differences could explain the discrepancy between fasting plasma glucose between the two studies.

Previous analysis of results from oral glucose tolerance testing in the rats from Study 1 revealed that CLA treatment had no effect on glucose tolerance in the lean rats (Diakiw, 2005). In the *fa/fa* rats, all diets that included the t10,c12 CLA isomer significantly reduced AUCg, representing an average improvement in glucose tolerance of 24.7% (Table 12). The c9,t11 isomer did not have a beneficial effect on glucose tolerance in the *fa/fa* rats in our study.

The beneficial effect of CLA, and more specifically the t10,c12 isomer, on glucose tolerance is in agreement with previous results found in rats. After only 2 weeks of feeding a 1.5% (wt/wt) CLA mixture added to 5% (wt/wt) corn oil, male

ZDF rats had improved glucose tolerance as assessed using AUCg (Houseknecht et al., 1998). Likewise, 7 week-old male ZDF rats fed a high-fat diet (total fat 40% (wt/wt)) supplemented with 1.5% (wt/wt) of a 50:50 mixture of the c9,t11 and t10,c12 isomers for 11 days had a significantly lower serum glucose response after an intraperitoneal injection of D-glucose (Ryder et al., 2001). The same amount of the c9,t11 isomer alone fed for the same period of time did not produce the same effect, suggesting that the t10,c12 isomer improves glucose tolerance in these rats. Henriksen et al. (2003) also found isomer-specific effects of CLA on glucose tolerance, as t10,c12 CLA given at 1.5 g/kg body weight to female *fa/fa* Zucker rats for 3 weeks resulted in an 11% reduction in AUCg, while treatment with the c9,t11 isomer alone and a 50:50 mixture of c9,t11 and t10,c12 CLA produced no change in glucose tolerance.

The liver is responsible for 1/3 of glucose uptake after an oral glucose load. Uptake by adipose tissue and skeletal muscle accounts for an additional 1/3, while the remaining 1/3 is taken up by non-insulin-dependent tissues (Meyer et al., 2002). Therefore, the improved response during an OGTT observed upon feeding with the t10,c12 isomer may be due in part to improved glucose uptake by adipose tissue, skeletal muscle, or the liver. Indeed, CLA, and more specifically the t10,c12 isomer, has been shown to improve insulin-stimulated glucose transport in skeletal muscle (Henriksen et al., 2003). However, since hepatic glucose uptake also constitutes a major portion of glucose disposal, determining if t10,c12 CLA also improves hepatic glucose uptake is an important

future research objective. Unlike skeletal muscle and adipose tissue, the predominant hepatic glucose transporter is GLUT2, which is not insulin dependent. GLUT2 is constitutively expressed on the plasma membrane of hepatocytes and has a low affinity and high capacity for glucose; thus, glucose flux across the hepatocyte membrane is determined by the relative glucose concentrations inside and outside of the cell (Olson & Pessin, 1996). Within the hepatocyte, glucose is phosphorylated to form glucose 6-phosphate by glucokinase, which also has a high capacity for glucose. The properties of GLUT2 and glucokinase result in rapid uptake of glucose by the hepatocyte for phosphorylation when portal glucose concentrations rise. The amount and activity of glucokinase is a major determinant of hepatic glucose uptake.

Transcriptional regulation of glucokinase is influenced by insulin; however, short-term regulation of glucokinase activity occurs via the glucokinase regulatory protein, a 68 kDa protein located in the nucleus of hepatocytes (Gregori et al., 2006; Van Schaftingen et al., 1994). In fasted conditions, glucokinase is sequestered in the nucleus of the hepatocyte by the glucokinase regulatory protein. In the fed state, glucokinase is separated from its regulatory protein, and translocates to the cytosol for phosphorylation of glucose. Thus, any alteration in glucokinase translocation would result in reduced hepatic glucose uptake. In pre-diabetic ZDF rats, glucokinase translocation is impaired (Fujimoto et al., 2004). Since *fa/fa* Zucker rats are also in a "pre-diabetic" state of hyperinsulinemia and impaired glucose tolerance, it is possible that glucokinase translocation is also impaired in these animals. Therefore, the beneficial effects

of t10,c12 CLA on the response to an oral glucose load might be mediated by improved glucokinase translocation.

Liver Function

ALT is an aminotransferase enzyme that is required for transamination reactions in the liver. Damage to the hepatocyte causes ALT to leak into the blood. Thus, elevated serum concentrations of ALT are indicative of liver dysfunction (McCullough, 2004). At the end of the current study, serum ALT concentrations were 7.3-fold higher in the control *fa/fa* Zucker rats compared to the control lean Zucker rats, suggesting compromised liver function in the *fa/fa* genotype. Feeding diets that contained the t10,c12 isomer improved liver function in *fa/fa* rats, as serum ALT concentrations of the 10-12, TOG, BIO and NCK groups were almost half that of the CTL group (Figure 21). This beneficial effect of t10,c12 CLA was unable to restore liver function to levels found in 6-week-old baseline *fa/fa* rats, as serum ALT was still 40% higher in the groups fed t10,c12 CLA, alone or in combination with other CLA isomers, compared to their baseline counterparts. The c9,t11 isomer did not appear to have a beneficial effect on liver function in our study.

Studies have shown previously that CLA can improve liver function in the *fa/fa* Zucker rat; however, the current study is the first to provide evidence that t10,c12 CLA, not c9,t11 CLA, improves liver function in this rat model. Noto et al. (2006) observed a 54% reduction in serum ALT concentrations in male *fa/fa* Zucker rats fed a 1.5% (wt/wt) CLA mixture for 8 weeks. In addition, serum

alkaline phosphatase, another marker of liver dysfunction, was reduced 64% with CLA feeding, further confirming the ability of CLA to improve liver function. Further evidence for a beneficial effect of CLA on liver function comes from a study by Nagao et al. (2005), who discovered that 8 weeks of dietary CLA given to male *fa/fa* Zucker rats reduced serum concentrations of ALT, aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase (3 additional serum markers of liver dysfunction) by 64.4%, 59.3%, 44.9%, and 58.3%, respectively. Furthermore, there was a strong inverse correlation between these serum markers of liver function and serum concentrations of adiponectin. This same study found that CLA feeding raised plasma concentrations of adiponectin; therefore, the improvement of liver function observed with CLA feeding in the current study could be due to the ability of CLA to increase plasma adiponectin concentrations. However, correlation analysis in the current study revealed that there was only a slight association between serum adiponectin and ALT concentrations ($r=0.027$, $p<0.0031$), suggesting that higher serum adiponectin concentrations with dietary t10,c12 CLA could not solely account for the reduction in serum ALT concentrations, and likely did not play a major role in this reduction.

Inflammation

Haptoglobin is a glycoprotein synthesized and secreted by the liver as part of the acute phase response (Bensi et al., 1985). In the rat, haptoglobin is a more sensitive indicator of inflammation than C-reactive protein (Giffen et al.,

2003). By the end of the 8 week study, *fa/fa* Zucker rats were in an inflammatory state: serum haptoglobin levels were 2.7-fold higher than lean controls. CLA feeding did not affect serum haptoglobin in the lean rats; however, *fa/fa* rats fed t10,c12 CLA, alone or in combination with other CLA isomers, significantly lowered serum haptoglobin (Figure 22). Interestingly, dietary c9,t11 CLA appeared to worsen serum haptoglobin concentrations, but this elevation was not statistically significant. These results support the hypothesis that the t10,c12 isomer reduces inflammation in the *fa/fa* Zucker rat. To the author's knowledge, this is the first study to report that dietary t10,c12 CLA reduces inflammation associated with obesity and MetS.

Although haptoglobin is regarded as a clinical marker of the hepatic acute-phase response, a recent study conducted by Chiellini et al. (2004) reported that serum haptoglobin level can also be considered a marker of adiposity in humans. They also reported a strong positive correlation between serum haptoglobin and both BMI and body fat mass. In addition, haptoglobin mRNA and protein could be detected in white adipose tissue from participants of the study, suggesting that haptoglobin is also synthesized by adipose tissue. In both *ob/ob* and *db/db* mice, mRNA levels of haptoglobin in white adipose tissue are higher than in wild-type mice (Chiellini et al., 2002). Therefore, elevated serum haptoglobin observed in obesity is either due to increased hepatic production, increased adipose production, or possibly a combination of both. Unfortunately, we cannot differentiate between attenuation of the acute-phase response to inflammation or

a reduction in haptoglobin mRNA in adipose tissue as the underlying mechanism behind reduced serum haptoglobin observed with dietary t10,c12 CLA in the current study. Gene expression of other adipocytokines has been shown to be affected by CLA; therefore adipose haptoglobin expression could also be modulated in this manner by CLA (Yurkova et al., 2004). Future research in this area should examine the effect of t10,c12 CLA on haptoglobin gene expression in the liver and in adipose tissue to determine the site of action of CLA on haptoglobin synthesis. As well, the relative contributions of hepatic vs. adipose production of haptoglobin in the obese *fa/fa* Zucker rat should also be determined.

There is limited research in animal models of obesity and insulin resistance with regards to how CLA affects the acute-phase response to inflammation. However, observational studies in humans have examined the effects of CLA supplementation on plasma levels of C-reactive protein, another inflammatory acute phase protein. Supplemental CLA, particularly the t10,c12 isomer, at 3 g/day to 4.2 g/day in both healthy and obese patients has been shown to increase plasma C-reactive protein (Smedman et al., 2005; Risérus et al., 2002b). These isomer-specific results are opposite to what was found in the current study. The discrepancy is most likely due to a difference in CLA metabolism and/or sensitivity in the rat vs. the human. However, other human studies have found no adverse effects of CLA on C-reactive protein (Moloney et al., 2004). To date, there have been no studies reporting a beneficial effect of CLA on C-reactive protein in humans.

Regulatory Proteins Involved in Hepatic Lipid Metabolism

i. AMPK

AMP-activated protein kinase (AMPK) is a sensor of cellular energy status, present in all eukaryotic cells, that responds to changes in the intracellular AMP:ATP ratio (Carling, 2004). When activated by phosphorylation of the α-subunit, AMPK serves to increase intracellular energy status by inhibiting reactions requiring ATP (for example, inhibiting de novo lipid synthesis by inhibition of ACC) (Hardie et al., 2003; Lochhead et al., 2000). Adiponectin has been shown to activate AMPK in hepatocytes (Yamauchi et al., 2002); therefore, we hypothesized that higher serum adiponectin concentrations observed with CLA-feeding mediates the reduction in hepatic steatosis by increasing activation of AMPK.

At the end of study 1, fasting levels of hepatic AMPK were significantly lower in the *fa/fa* rats compared to the lean rats. Dietary CLA had no effect on fasting hepatic AMPK in the *fa/fa* rats (Figure 23). While the mean fasting hepatic AMPK level of the ln 10-12, ln TOG, ln BIO, and ln NCK groups was lower than those of the ln 9-11 and ln CTL groups, these differences did not reach statistical significance due to large standard error. At the end of study 2, there was a trend ($p=0.0575$) for fed state hepatic AMPK to be lower in the fa 10-12 fed group (Figure 33). This observation might be seen as a negative effect of t10,c12 CLA, as reducing the amount of AMPK protein reduces the amount available for activation, potentially lowering fatty acid oxidation in the liver of these rats.

Activation of AMPK was also measured in both the fed and fasted states. As mentioned previously, it was hypothesized that dietary CLA would raise serum adiponectin concentrations and consequently raise levels of hepatic pAMPKα. Based on means alone, it appeared that fasting AMPK activation was higher in lean rats compared to *fa/fa* rats (Figure 24). However, due once again to large standard errors this apparent difference was not statistically significant, suggesting that obesity and insulin resistance in the *fa/fa* Zucker rat do not affect AMPK activation in the fasted state. Activation of AMPK is dependent on nutritional state; activation is higher in fasted states when cellular energy status is low, and declines in the fed state when cellular energy status is high (Carling, 2004). Previous studies examining the effect of PUFAs on AMPK activation after a meal have found less reduction in AMPK activation in rats fed a PUFA-containing meal vs. a control meal, resulting in a net higher AMPK activation in the fed state (Suchancova et al., 2005). Thus, it seemed logical to check for reductions in AMPK activation in *fa/fa* Zucker rats after they had been fed a CLA-containing meal. Results from Study 2 show that fed-state levels of pAMPKα were not affected by CLA-feeding (Figure 34). This lack of effect indicates that reduced hepatic lipid content observed with dietary t10,c12 CLA occurs independent of AMPK activation. Unexpectedly, nutritional status did not affect AMPK activation in the current study, as AMPK activation was not different between the fa CTL fed and the fa CTL fasted groups.

Despite the elevation in serum adiponectin observed with dietary t10,c12 CLA, there was no concomitant rise in AMPK activation in the liver of fed or

fasted rats. This lack of effect could be due to the inability of higher amounts of adiponectin to actually reach the liver and be transported into hepatocytes for activation of AMPK. Nagao et al. (2005) found no change in mRNA levels of both adiponectin receptors in the liver with dietary CLA. It is possible that increased amounts of adiponectin could not be transported into the liver due to an insufficient amount of adiponectin receptors on the hepatocyte membrane.

AMPK activation in the liver occurs only with full-length adiponectin (Yamauchi et al., 2002). It is possible that the higher levels of serum adiponectin observed upon feeding with t10,c12 increases are actually higher levels of the globular form of adiponectin, which would not produce an effect on AMPK activation in hepatocytes. However, Diakiw (2005) observed no effect of dietary CLA on activation of AMPK in skeletal muscle, in which AMPK activation occurs with both the full-length and globular form of adiponectin (Yamauchi et al., 2002). In smooth muscle cells, 60 μM of c9,t11 CLA elevated activation of AMPK, although this did not reach statistical significance (Zirk, 2005). The RIA that we used to quantify serum adiponectin does not identify the recognition sequence in the adiponectin protein that is identified by the adiponectin antibody, making it impossible to tell if the assay detects either form or only the full-length form. Future research should focus on the potential for CLA to affect not only quantity of adiponectin, but also the form of adiponectin.

ii. SREBP-1

SREBP-1 is a 125 kDa transcription factor regulating transcription of genes involved in lipid synthesis. In its inactive form, SRBEP-1 is embedded in the endoplasmic reticular membrane. Upon activation, the full length form undergoes two proteolytic cleavages to produce a 68 kDa fragment that translocates to the nucleus for initiation of transcription (Brown et al., 2000). Previous research with CLA-feeding in mice has shown that reduced insulin resistance and hepatic steatosis observed with dietary c9,t11 CLA is associated with reduced activation of SREBP-1 (Roche et al., 2002). Therefore, we hypothesized that t10,c12 CLA would reduce insulin resistance and hepatic steatosis via a mechanism involving suppression of SREBP-1 activation.

In the fasted state, *fa/fa* Zucker rats had significantly lower levels of hepatic full-length SREBP-1 (Figure 25). Dietary CLA did not change fasting levels of hepatic full length SREBP-1. However, in the fed state, there was a strong trend for hepatic full-length SREBP-1 to be significantly higher in both the groups fed c9,t11 and t10,c12 CLA (Figure 35). Initially this may appear to be a negative result, as it suggests that dietary CLA promotes lipid synthesis in the liver through increased gene expression of a lipogenic transcription factor. Both glucose and insulin positively regulate SREBP-1 transcription in the liver (Hasty et al., 2000). Therefore, higher SREBP-1 gene expression in the liver of rats fed c9,t11 and t10,c12 could result from improved hepatic insulin and/or glucose signaling.

Transcription of lipogenic enzymes occurs when full-length SREBP-1 is cleaved to produce the active 68 kDa SREBP-1 fragment, which makes measuring the active form of SREBP-1 important to assess its possible role in the anti-steatotic effects of CLA. In the fasted state, SREBP-1 activation did not differ between lean and *fa/fa* rats, and dietary CLA had no effect on SREBP-1 activation (Figure 26). In the fed state, there was no effect of dietary CLA on SREBP-1 activation (Figure 36). This implies that reduced hepatic lipid content observed with dietary t10,c12 does not occur through reduced activation of SREBP-1.

A limited number of studies have examined how CLA affects SREBP-1 activation, but have provided conflicting results. In bovine mammary epithelial cells, treatment with t10,c12 CLA significantly reduced SREBP-1 activation, with a simultaneous reduction in ACC and FAS mRNA levels (Peterson et al., 2004). However, although protein levels of mature SREBP-1 were not measured, Takahashi et al. (2003) observed elevated mRNA levels of ACC and FAS (indirect markers of SREBP-1 activation) in livers of C57BL/6J and ICR mice fed a 1.5% (wt/wt) CLA mixture for 3 weeks. Likewise, Noto et al. (2006) found higher mRNA levels of ACC in *fa/fa* Zucker rats fed a 1.5% (wt/wt) CLA mixture for 8 weeks. Once again, however, activation of SREBP-1 was not directly measured in this study. Clearly, more research studies are required to clarify the potential role of CLA in SREBP-1 activation.

SUMMARY AND CONCLUSIONS

Major Research Findings

In 6-week-old *fa/fa* Zucker rats, elevated hepatic lipid concentration and serum ALT are present, suggesting hepatic insulin resistance and liver dysfunction occur early in these rats.

In 14-week-old lean Zucker rats, dietary supplementation with CLA isomers for 8 weeks had no effect on hepatic weights, hepatic lipid concentration, serum ALT, or serum haptoglobin.

In 14-week-old *fa/fa* Zucker rats, dietary supplementation with CLA isomers for 8 weeks had the following effects:

Hepatic Weights and Steatosis

- Dietary t10,c12 CLA, alone or in combination with other CLA isomers, reduced absolute and relative liver weights in *fa/fa* Zucker rats. Dietary c9,t11 CLA had no effect on liver weights.
- Dietary t10,c12 CLA, alone or in combination with other CLA isomers, dramatically reduced hepatic steatosis in the *fa/fa* Zucker rats, while c9,t11 did not have any effect. The effect of dietary t10,c12 CLA was

so strong that lipid concentrations were reduced to levels lower than those of 6-week-old *fa/fa* Zucker rats.

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Serum ALT

- Dietary t10,c12 CLA, alone or in combination with other CLA isomers, reduced serum ALT concentrations in *fa/fa* Zucker rats. Dietary c9,t11 CLA did not lower serum ALT in *fa/fa* Zucker rats.

Serum Haptoglobin

- In *fa/fa* Zucker rats, dietary t10,c12 CLA, alone or in combination with other CLA isomers, reduced serum haptoglobin. Dietary c9,t11 CLA did not have this beneficial effect.

Hepatic AMPK and pAMPKα

- In the fasted state, hepatic AMPK levels were significantly lower in *fa/fa* Zucker rats than in lean Zucker rats. Dietary CLA isomers had no effect on hepatic AMPK levels in lean and *fa/fa* Zucker rats in both the fed and fasted states.
- In the fasted state, lean and *fa/fa* Zucker rats did not differ in respect to hepatic pAMPKα levels, and there was no effect of dietary CLA isomers on pAMPKα levels. Likewise in the fed state, there was no difference in hepatic pAMPKα levels between the lean and *fa/fa* Zucker rats, and CLA-feeding had no effect.

Hepatic SREBP-1 p125 and SREBP-1 p68

- In the fasted state, hepatic SREBP-1 p125 levels were significantly lower in *fa/fa* Zucker rats compared to lean Zucker rats. There was no effect of dietary CLA isomers on hepatic SREBP-1 p125 levels. In the fed state, there was a trend for SREBP-1 p125 to be higher in *fa/fa* Zucker rats fed c9,t11 CLA.
- In both the fasted and fed states, hepatic SREBP-1 p68 was not different between lean and *fa/fa* Zucker rats. Dietary CLA had no effect on hepatic SREBP-1 p68 levels in either lean or *fa/fa* Zucker rats.

For those parameters which were improved with feeding t10,c12 CLA, the improvement was not sufficient to restore to values found in lean Zucker rats of the same age, or to values found in 6-week old *fa/fa* Zucker rats. The only exception is liver lipid concentration, which was reduced almost to levels seen in lean Zucker rats, and was reduced to levels lower than that of 6-week-old *fa/fa* Zucker rats.

The magnitude of improvement of parameters observed with feeing t10,c12 CLA was similar for all diets containing the t10,c12 CLA isomer. More specifically, beneficial changes elicited by the NCK diet (which also contained t8,c10 and c11,t13 CLA) were not significantly different than the changes elicited by other diets containing t10,c12 CLA, suggesting that CLA isomers in combination do not act synergistically.

Overall, the beneficial effects of dietary CLA on hepatic steatosis, liver dysfunction, and inflammation associated with MetS can be attributed to t10,c12 CLA. However, this attenuation of hepatic steatosis is not a result of increased AMPK activation or suppression of SREBP-1 activation in the liver. Although results from the current study imply that dietary t10,c12 CLA is a potential dietary therapeutic option for treatment of MetS, results must be taken with caution. First, extrapolation of results from animal models, especially rodents, must be done with extreme caution. Second, amounts of t10,c12 CLA in food products are relatively low compared to those of the c9,t11 isomer, which was found to be neutral in the current study. If further CLA research reveals that t10,c12 CLA is beneficial in humans, supplements containing t10,c12 CLA will be necessary to ingest amounts sufficient to reap beneficial effects. Alternatively, methods of increasing the t10,c12 CLA content in dairy and meat products will have to be developed.

Strengths and Limitations

Strengths

- Diets were formulated to contain the same amount [0.4% (wt/wt)] of c9,t11 and/or t10,c12), even in diets containing the commercial CLA mixtures.
- Examining levels of SREBP-1 and AMPK in both the fed and fasted state allowed for a more complete picture on how CLA affects hepatic lipid metabolism.
- An 8 week feeding trial is relatively long in duration, which allowed for assessment of the long-term effects of dietary CLA on MetS.

Limitations

- The cause of obesity in the *fa/fa* Zucker rat, the mutation of the leptin receptor, is not reflective of the major cause of obesity in developed countries.
- In order to consume the same dose of CLA as was used in this study (at least 0.4% (wt/wt) CLA) a human would have to consume an unrealistic amount of dairy and/or meat products. Furthermore, food sources of CLA contain small amounts of the t10,c12 isomer, the isomer that was observed to have beneficial effects in our study.
- Only one dose of 0.4% wt/wt was used in our studies; therefore we cannot comment on possible dose-response effects of dietary CLA.

- Measurements were made after only one time point (8 weeks). The beneficial effects that we observed may manifest themselves at an earlier time point. In addition, further improvement in parameters that we measured may occur with a longer feeding trial.

Areas for Future Research

- Investigating the effects of CLA isomers on MetS and hepatic steatosis in a diet-induced model of obesity, such as the high-fat or high-sucrose fed Sprague Dawley rat.
- Determining the relative contribution of hepatic glucose uptake in the improved response to an oral glucose dose, focusing on activity of glucokinase and glucokinase regulatory protein, and levels of GLUT2 in the hepatocyte.
- Histological examination of livers from *fa/fa* Zucker rats fed CLA isomers and control diet to assess degree of amelioration of macrovesicular vs. microvesicular lipid accumulation. This would also reveal the extent of steatohepatitis, if any, in the *fa/fa* Zucker rat.
- Determining if CLA isomers affect mRNA levels or activation of ChREBP or Foxa2, other hepatic transcription factors involved in transcription of both glucose-metabolising and lipid-metabolizing enzymes.
- Determining the mechanism for reduced serum cholesterol seen with t10,c12 CLA feeding, including examining possible effects of CLA on SREBP-2, the transcription factor involved in transcription of genes involved in cholesterol biosynthesis.
- Determining the mechanism for reduced hyperleptinemia elicited by t10,c12 CLA, with a particular focus on how t10,c12 CLA influences

leptin feedback mechanisms and signaling pathways in adipocytes, hepatocytes and skeletal muscle.

REFERENCES

Abu-Elheiga, L., Oh, W., Kordari, P., & Wakil, S. J. (2001). Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science, 291*, 2613-2616.

Aguirre, V., Tohru, U., Yenush, L., Davis, R., & White, M. F. (2000). The c-Jun NH_2-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of ser^{307}. *Journal of Biological Chemistry, 275*, 9047-9054.

Akahoshi, A., Goto, Y., Murao, K., Miyazaki, T., Yamasaki, M., Nonaka, M., Yamada, K., & Sugano, M. (2002). Conjugated linoleic acid reduces body fats and cytokine levels of mice. *Bioscience, Biotechnology, and Biochemistry, 66*, 916-920.

Alberti, F. G. M. M. & Zimmet, P. Z. (1998). For the WHO consultation. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus, provisional report of a WHO consultation. *Diabetic Medicine, 15*, 539-553.

Allain, C. C., Poon, L., Chan, S. G., Richmond, W., & Fu, P. (1974). Enzymatic determination of total serum cholesterol. *Clinical Chemistry, 20*, 470-475.

Altomonte, J., Harbaran, S., Richter, A., & Dong, H. (2003). Fat depot-specific expression of adiponectin is impaired in Zucker fatty rats. *Metabolism, 52*, 958-963.

American College of Endocrinology. (2003). American Association of Clinical Endocrinology position statement on insulin resistance syndrome. *Endocrine Practice, 9*, 327-252.

Aminot-Gilchrist, D. V. & Anderson, H. D. (2004). Insulin resistance-associated cardiovascular disease: potential benefits of conjugated linoleic acid. *American Journal of Clinical Nutrition, 79*(Suppl), 1159S-1163S.

Angelico, F., Del Ben, F., Conti, R., Francioso, S., Feole, K., Maccioni, D, Antonini, T. M., & Alssandri, C. (2003). Non-alcoholic fatty liver syndrome: a hepatic consequence of common metabolic diseases. *Journal of Gastroenterology and Hepatology, 18*, 588-594.

Angulo, P., Keach, J. C., Batts, K. P., & Lindor, K. D. (1999). Independent predictors of liver fibrosis in patients with non-alcoholic steatohepatitis. *Hepatology, 30*, 1356-1362.

Arita, Y., Kihara, S., Ouchi, N., Takahashim M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaokam K., Kuriyama, H., Nishida, M., Funahashi, T., & Matsuzawa, Y. (1999). Paradoxical decrease in an adipose-specific protein, adiponectin, in obesity. *Biochemical and Biophysical Research Communications, 257*, 79-83.

Armario, A., Martí, J., & Gil., M. (1990). Glucose response to acute stress is sensitive to the intensity of the stressor and to habituation. *Psychoneuroendocrinology, 15*, 341-347.

Armario, A., Montero, J. L,. & Balasch, J. (1986). Sensitivity of corticosterone and some metabolic variables to graded levels of low intensity stress in adult male rats. *Physiology & Behaviour, 37*, 559-561.

Aygun, C., Senturk, O., Hulagu, S., Uraz, S., Celebi, A., Konduk, T., Mutlum B., & Zeynep, C. (2006). Serum levels of hepatoprotective peptide adiponectin in non-alcoholic fatty liver disease. *European Journal of Gastroenterology and Hepatology, 18*, 175-180.

Azuma, K., Katsukawa, F., Oguchi, S., Murata, M., Yamazaki, H., Shimada, A., & Saruta, T. (2003). Correlation between serum resistin level and adiposity in obese individuals. *Obesity Research, 11*, 997-1001.

Balkau, B. & Charles, M. A. (1999). Comment on the provisional report from the WHO consultation. European Group for the Study of Insulin Resistance (EGIR). *Diabetic Medicine, 16*, 442-443.

Banni, S. (2002). Conjugated linoleic acid metabolism. *Current Opinion in Lipidology, 13*, 261-266.

Bauman, D. E., Baumgard, L. H., Corl. B. A., & Griinari, J. M. (1999). Biosynthesis of conjugated linoleic acid in ruminants. Proceedings of the American Society of Animal Science [On-line]. Available: http://www.asas.org/jas/symposia/proceedings/0937.pdf.

Belury, M. (2002). Dietary conjugated linoleic acid in health: physiological effects and mechanisms of action. *Annual Review of Nutrition, 22*, 505-531.

Bensi, G., Raugei, G., Klefenz, H., & Cortese, R. (1985). Structure and expression of the human haptoglobin locus. *EMBO Journal, 4*, 119–126.

Berg, A. H., Combs, T. P., & Scherer, P. E. (2002). ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends in Endocrinology and Metabolism, 13*, 84-89.

Berger, J., Bailey, P., Biswas, C., Cullinan, C. A., Doebber, T. W., Hayes, N. S., Saperstein, R., Smith, R. G., & Leibowits. M. D. (1996). Thiazoledinediones produce a conformational change in peroxisome proliferator-activated receptor-γ: binding and activation correlated with antidiabetic actions in *db/db* mice. *Endocrinology, 137*, 4189-4195.

Bergeron, R., Previs. S. F., Cline, G. W., Perret, P., Russell, R. R. 3rd, Young, L. H., & Shulman, G. I. (2001). Effect of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats. *Diabetes, 50*, 1076-1082.

Beylot, M., Pinteur, C., & Peroni, O. (2006). Expression of the adiponectin receptors AdipoR1 and AdipoR2 in lean rats and in obese Zucker rats. *Metabolism, 55*, 396-401.

Bluher, M., Fasshauer, M., Tonjes, A., Kratzsch, J., Schon, M. R., & Paschke, R. (2005). Association of interleukin-6, C-reactive protein, interleukin-10 and adiponectin plasma concentrations with measures of obesity, insulin sensitivity and glucose metabolism. *Experimental and Clinical Endocrinology & Diabetes, 113*, 534-537.

Bogaka, I., Roane, D. S., Xi, X., Zhou, J., Li, B., Ryan, D. H., & Martin, R. J. (2004). Expression of genes likely involved in glucose sensing in the obese rat brain. *Nutritional Neuroscience, 7*, 67-74.

Brown, M. S., Ye, J., Rawson, R. B., & Goldstein, J. L. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell, 100*, 391-398.

Brunt, E. M. (2004). Nonalcoholic steatohepatitis. *Seminars in Liver Disease, 24*, 3-20.

Brunt, E. M., Neuschwander-Tetri, B. A., Oliver, D., Wehmeier, K. R., & Bacon, B. R. (2004). Nonalcoholic steatohepatitis: histologic features and clinical correlations with 30 blinded biopsy specimens. *Human Pathology, 35*, 1070-1082.

Bugianesi, E., Gentilcore, E., Manini, R., Natale, S., Vanni, E., Villanova, N., David, E., Rizzetto, M., & Marchesini, G. (2005a). A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease. *American Journal of Gastroenterology, 100*, 1082-1090.

Bugianesi, E., Pagotto, U., Manini, R., Vanni, E., Gastaldelli, A., di Iasio, R., Gentilcore, E., Natale, S., Cassader, M., Rizetto, M., Pasquali, R., & Marchesini, G. (2005b). Plasma adiponectin in non-alcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, but no to liver disease severity. *The Journal of Clinical Endocrinology and Metabolism, 90*, 3498-3504.

Bugianesi, E., Zannoni, C., Vanni, E., Marzocchi, R., & Marchesini, G. (2004). Non-alcoholic fatty liver and insulin resistance: a cause-effect relationship? *Digestive and Liver Disease, 36*, 165-173.

Buhl, E. S., Jessen, N., Pold, R., Ledet, T., Flyvbjerg, A., Pedersen, S. B., Pedersen, O., Schmitz, O., & Lund, S. (2002). Long-term AICAR administration reduces metabolic disturbances and lowers blood pressure in rats displaying features of the insulin resistance syndrome. *Diabetes, 51*, 2199-2206.

Carling, D. (2004). The AMP-activated protein kinase cascade-a unifying system for energy control. *Trends in Biochemical Sciences, 29*, 18-24.

Cheung, A. T., Ree, D., Kolls, J. K., Fuselier, J., Choy, D. A., & Bryer-Ash, M. (1998). An *in vivo* model for elucidation of the mechanism of tumor necrosis factor-α (TNF-α)-induced insulin resistance: evidence for differential regulation of insulin signalling by TNF-α. *Endocrinology, 139*, 4928-4935.

Chevenne, D., Trivin, F., & Porquet, D. (1999). Insulin assays and reference values. *Diabetes and Metabolism*, 25, 459-476.

Chiellini, C., Bertacca, A., Novelli, S. E., Gorgun, C. Z., Ciccarone, A., Giordano, A., Xu, H., Soukas, A., Costa, M., Gandini, D., Dimitri, R., Bottone, P., Cecchetti, P., Pardini, E., Perego, L., Navalesi, R., Folli, F., Benzi, L., Cinti, S., Friedman, J. M., Hotamisligil, G. S., & Maffei, M. (2002). Obesity modulates the expression of haptoglobin in the white adipose tissue via TNFalpha. *Journal of Cellular Physiology, 190*, 251-258.

Chiellini, C., Santini, F., Marsili, A., Berti, P., Bertacca, A., Pelosini, C., Scartabelli, G., Pardini, E., Lopez-Soriano, J., Centoni, R., Ciccarone, A. M., Benzi, L., Vitti, P., Del Prato, S., Pinchera, A., & Maffei, M. (2004). Serum haptoglobin: a novel marker of adiposity in humans. *Journal of Clinical Endocrinology and Metabolism, 89*, 2678-2683.

Chua, S. C. Jr., White, D. W., Wu-Peng, X. S., Liu, S. M., Okada, N., Kershaw, E. E., Chung, W. K., Power-Kehoe, L., Chua, M., Tartaglia, L. A., & Leibel, R. L. (1996). Phenotype of fatty due to Gln269Pro mutation in the leptin receptor (Lepr). *Diabetes, 45*, 1141-1143.

Cleeman, J. I. (2003). Executive summary of the third report of the National Cholesterol Education Program (NCEP) Expert panel on detection, evaluation and treatment of high cholesterol. *Journal of the American Medical Association, 285*, 2486-2497.

Clément, L., Poirier, H., Niot, I., Bocher, V., Guerre-Millo, Krief, S., Staels, B., & Besnard, P. (2002). Dietary trans-10, cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. *Journal of Lipid Research, 43*, 1400-1409.

Coimbra, T. M., Janssen, U., Grone, H. J., Ostendorf, T., Kunter, U., Schmidt, H., Brabant, G., & Floege, J. (2000). Early events leading to renal injury in obese Zucker (fatty) rats with type II diabetes. *Kidney International, 57*, 167-182.

Combs, T. P., Berg, A. H., Obici, S., Scherer, P. E., & Rossetti, L. (2001). Endogenous glucose production is inhibited by the adipose-derived Acrp30. *Journal of Clinical Investigation, 108*, 1875-1881.

Comert, B., Mas, M. R., Erdem, H., Dinc, A., Saglamkaya, U., Cigerim, M., Kurhan, O., Unal, T., & Kocabalkem, F. (2001). Insulin resistance in non-alcoholic steatohepatits. *Digestive and Liver Disease, 33*, 353-358.

Considine, R. V., Sinha, M. K., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Nyce, M. R., Ohannesian, J. P., Marco, C. C., McKee, L. J., Bauer, T. L., & Caro, J. F. (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine, 334*, 292-295.

Cook, S., Weitzman, M., Auinger, P., Nguyen, M., & Dietz, W. H. (2003). Prevalence of a metabolic syndrome phenotype in adolescents: findings from the third National Health and Nutrition Examination Survey, 1998-1994. *Archives of Pediatric and Adolescent Medicine, 157*, 821-827.

Corl, B. A., Barbano, D. M., Bauman, D. E., & Ip, C. (2003). *cis*-9, *trans*-11 CLA derived endogenously from *trans*-11 18:1 reduces cancer risk in rats. *Journal of Nutrition, 133*, 2893–2900.

Corl, B. A., Baumgard, L. H., Dwyer, D. A., Griinari, J. M., Phillips, B. S., & Bauman, D. E. (2001). The role of delta(9)-desaturase in the production of *cis*-9, *trans*-11 CLA. *Journal of Nutritional Biochemistry, 12*, 622-630.

Daval, M., Foufelle, F., & Ferre, P. (2006). Functions of AMP-activated protein kinase in adipose tissue. *Journal of Physiology*, May 18, 2006 (E-pub ahead of print).

de Alvaro, C., Teruel, T., Hernandez, R., & Lorenzo, M. (2004). Tumor necrosis factor α produces insulin resistance in skeletal muscle by activation of inhibitor κB kinase in a p38 MAPK-dependent manner. *Journal of Biological Chemistry, 279*, 17070-17078.

de Boer, S. F., Koopmans, S. J., Slangen, J. L., & van der Gugten, J. (1990). Plasma catecholamine, corticosterone and glucose responses to repeated stress in rats: effect of interstressor interval length. *Physiology & Behaviour, 47*, 1117-1124.

Debril, M.-B., Renaud, J.-P., Fajas, L., & Auwerx, J. (2001). The pleiotropic functions of peroxisome proliferator-activated receptor. *Journal of Molecular Medicine, 79*, 30-47.

Degawa-Yamauchi, M., Bovenkerk, J. E., Juliar, B. E., Watson, W., Kerr, K., Jones, R., Zhu, Q., & Considine, R. V. (2003). Serum resistin (FIZZ3) protein is increased in obese humans. *Journal of Clinical Endocrinology and Metabolism, 88,* 5452-5455.

Del Aguila, L., Claffey, K. P., & Kirwan, J. P. (1999). TNF-α impairs insulin signaling and insulin stimulation of glucose uptake in C2C12 muscle cells. *American Journal of Physiology: Endocrinology and Metabolism, 276,* E849-E855.

den Boer, M., Voshol, P. J., Kuipers, F., Havekes, L. M., & Romijn, J. A. (2004). Hepatic steatosis: a mediator of the metabolic syndrome. Lessons from animal models. *Arteriosclerosis Thrombosis and Vascular Biology, 24*, 644-649.

Desvergne, B. & Wahli, W. (1999). Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocrine Reviews, 20*, 649-688.

Dhiman, T. R., Nam, S. H., & Ure, A. L. (2005). Factors affecting conjugated linoleic acid content in milk and meat. *Critical Reviews in Food Science and Nutrition, 45*, 463-482.

Diakiw, R. P. (2005). Effects of specific conjugated linoleic acid (CLA) isomers on insulin resistance, and skeletal muscle AMP-activated protein kinase-alpha (AMPK-alpha) in *fa/fa* and lean Zucker rats [dissertation]. Winnipeg (Manitoba): University of Manitoba.

Dobson, G. (2002). Analysis of fatty acids in functional foods with emphasis on ω3 fatty acids and conjugated linoleic acid. In J. Hurst (Ed.), *Methods of Analysis for Functional Foods and Nutraceuticals* (pp. 65-99). U. S. A.: CRC Press.

Duncan, G. E., Li, S. M., & Zhou, X. H. (2004). Prevalence and trends of a metabolic syndrome phenotype among U.S. adolescents, 1999-2000. *Diabetes Care, 27*, 2438-2443.

Epstein, F. H. (2000). Cytokines in alcoholic and non-alcoholic steatohepatitis. *New England Journal of Medicine, 343*, 1467-1476.

Evans, R. M. (2004). 2003 Keio Medical Science Prize commemorative lecture. PPARs and the complex journey to obesity. *Keio Journal of Medicine, 53*, 53-58.

Fagot-Campagna, A., Pettitt, D. J., Engelgau, M. M., Burrow, N. R., Geiss, L.S., Valdez, R., Beckles, G. L., Saaddine, J., Gregg, E. W., Williamson, D. F., & Narayan, K. M. (2000). Type 2 diabetes among North American children and adolescents: an epidemiologic review and a public health perspective. *Journal of Pediatrics,136*, 664-672.

Ferré, P. (2004). The biology of peroxisome proliferator activated receptors. Relationship with lipid metabolism and insulin sensitivity. *Diabetes, 53*(Suppl. 1), S43-S50.

Ford, E. S. (2004). Prevalence of the metabolic syndrome in US populations. *Endocrinology and Metabolism Clinics of North America, 33*, 333-350.

Forman, B. M., Chen, J., & Evans, R. M. (1997). Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. *Procedures of the National Academy of Science, 94*, 4312-4317.

Frederich, R. C., Lolmann, B., Hamann, A., Napolitano-Rosen, A., Kahn, B. B., Lowell, B. B., & Flier, J. S. (1995). Expression of ob mRNA and its encoded protein in rodents. Impact of nutrition and obesity. *Journal of Clinical Investigation, 96*, 1658-1663.

Gabbay, M., Cesarini, P.R., & Dib, S. A. (2003). Type 2 diabetes in children and adolescents: literature review. *Jornal de Pediatria, 79*, 201-208.

Gable, D. R., Hurel, S. J., & Humphries, S. E. (2006). Adiponectin and its gene variants as risk factors for insulin resistance, the metabolic syndrome and cardiovascular disease. *Atherosclerosis*, March 30, 2006 (E-pub ahead of print).

Gearing, A. J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., & Gordon, J. L. (1994). Processing of tumor necrosis factor-alpha precursor by metalloproteinases. *Nature, 370*, 555-557.

Gibbons, G. F. (1990). Assembly and secretion of hepatic very-low density lipoprotein. *Biochemistry Journal, 268*, 1-13.

Giffen, P. S., Turton, J., Andrews, C. M., Barrett, P., Clarke C. J., Fung, K.-W., Munday, M. R., Roman, I. F., Smyth, R., Walshe, K., & York, M. J. (2003). Markers of experimental acute inflammation in the Wistar Han rat with particular reference to haptoglobin and C-reactive protein. *Archives of Toxicology, 77*, 392-402.

Gillam, M. (2003). The effects of dietary Zn or n-3 fatty aicds on hyperinsulinemia, hyperlipidemia and pancreatic function in *fa/fa* and lean Zucker rats [dissertation]. Winnipeg (Manitoba): University of Manitoba.

Gonzalez, A. S., Guerrero, D. B., Soto, M. B., Diaz, S. P., Martinez-Olmos, M., & Vidal, O. (2006). Metabolic syndrome, insulin resistance and the inflammation markers C-reactive protein and ferritin. *European Journal of Clinical Nutrition*, February 22, 2006 (E-pub ahead of print).

Griinari, J. M., Corl, B. A., Lacy, S. H., Chouinard, P. Y., Nurmela, K. V. V., & Bauman, D. E. (2000). Conjugated linoleic acid in synthesized endogenously in lactating cows by delta(9)-desaturase. *Journal of Nutrition, 130*, 2285-2291.

Groff, J. L. & Gropper, S. S. Diabetes: metabolism out of control. In: Advanced *Nutrition and Human Metabolism* (pp.242-244). Belmont: Wadsworth /Thomas Learning

Gross, V., Andus, T., Castell, J., Vom Berg, D., Heinrich, P. C., & Gerok, W. (1989). O- and N- glycosylation lead to different molecular mass forms of human monocyte leukocyte interleukin-6. *FEBS Letters, 247*, 323-326.

Grundy, S. M. (2000). Metabolic complications of obesity. *Endocrine, 13*, 155-165.

Guerre-Millo, M. (2003). Extending the glucose/fatty acid cycle: a glucose/adipose tissue cycle. *Biochemical Society Transactions, 31*, 1161-1164.

Ha, Y. L., Grimm, N. K., & Pariza, M. W. (1987). Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. *Carcinogenesis, 8*, 1881-1887.

Haffner, S. M. (2006). The metabolic syndrome: inflammation, diabetes mellitus, and cardiovascular disease. *American Journal of Cardiology, 97*, 3A-11A.

Halpern, A. & Mancini, M. C. (2003). Treatment of obesity: an update on anti-obesity medications. *Obesity Reviews, 4*, 25-42.

Hara, T., Fujiwara, H., Shoji, T., Mimura, T., Nakao, H., & Fujimoto, S. (2003). Decreased plasma adiponectin levels in young obese males. *Journal of Atherosclerosis and Thrombosis, 10*, 234-238.

Hardie, G. D., Scott, J. W., Pan, D. A., & Hudson, E. R. (2003). Management of cellular energy by the AMP-activated protein kinase system. *FEBS Letters, 546*, 113-120.

Hargrave, K. M., Azain, M. J., Kachman, S. D., & Miner, J. L. (2003). Conjugated linoleic acid does not improve insulin tolerance in mice. *Obesity Research, 11*, 1104-1115.

Hassard, T. H. (1991). Analysis of variance. In *Understanding Biostatistics* (pp.75-97). St. Louis, MO: Mosby – Year Book.

Hasty, A. H., Shimano, H., Yahagi, N., Amemiya-Kudo, M., Perrey, S., Yoshikawa, T., Osuga, J., Okazaki, H., Tamura, Y., Iizuka, Y., Shionoiri, F., Ohashi, K., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., & Yamada, N. (2000). Sterol regulatory element-binding protein-1 is regulated by glucose at the transcriptional level. *Journal of Biological Chemistry, 275*, 31069-31077.

Heliovaara, M. K., Teppo, A. M., Karonen, S. L., Tuominen, J. A., & Ebeling, P. (2005). Plasma IL-6 concentration is inversely related to insulin sensitivity, and acute-phase proteins associate with glucose and lipid metabolism in healthy subjects. *Diabetes, Obesity & Metabolism, 7*, 729-736.

Henin, N., Vincent, M. F., Gruber, H. E., & Van den Berghe, G. (1995). Inhibition of fatty acid and cholesterol synthesis by stimulation of AMP-activated protein kinase. *FASEB Journal, 9*, 541-546.

Henriksen, E. J., Teachey, M. K., Taylor, Z. C., Jacob, S., Ptock, A., Kramer, K., & Hasselwander, O. (2003). Isomer-specific actions of conjugated linoleic acid on muscle glucose transport in the obese Zucker rat. *American Journal of Physiology: Endocrinology and Metabolism, 285*, E98-E105.

Hofmann, C., Lorenz, Braithwaite, S. S., Colca, J. R., Palazuk, B. J., Hotamisligil, G. S., & Spiegelman, B. M. (1994). Altered gene expression for tumor necrosis factor-alpha and its receptors during drug and dietary modulation of insulin resistance. *Endocrinology, 134*, 264-270.

Horton, J. D. (2002). Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. *Biochemical Society Transactions, 30*, 1091-1095.

Horton, J. D., Shimomura, I., Brown, M. S., Hammer, R. E., Goldstein, J. L., & Shimano, H. (1998). Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *Journal of Clinical Investigation, 101*, 2331-2339.

Hotamisligil, G. S., Murray, D. L., Choy, L. N., & Spiegelman, B. M. (1994). Tumor necrosis factor α inhibits insulin signaling from the insulin receptor. *Proceedings of the National Academy of Sciences, 91*, 4854-4854.

Hotamisligil, G. S., Shargill, N. S., & Spiegelman, B. M. (1993). Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science, 259*, 87-91.

Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwahashi, H., Kuriyama, H., Ouchi, N., Maeda, K., Nishida, M., Kihara, S., Sakai, N., Nakajima, T., Hasegawa, K., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Hanafusa, T., & Matsuzawa, Y. (2000). Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arteriosclerosis, Thrombosis, and Vascular Biology, 20*, 1595-1599.

Hotta, K., Funahashi, T., Bodkin, N. L., Ortmeyer, H. K., Arita, Y., Hansen, B. C., & Matsuzawa, Y. (2001). Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to Type 2 diabetes in rhesus monkeys. *Diabetes, 50*, 1126-1133.

Houseknecht, K. L., Vanden Heuvel, J. P., Moya-Camarena, S. Y., Portocarrero, C. P., Peck, L. W., Nickel, K. P., & Belury, M. A. (1998). Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker Diabetic Fatty *fa/fa* rat. *Biochemical and Biophysical Research Communications, 244*, 678-682.

Hurley, P. L., Anderson, K. A., Frazone, J. M., Kemp, B. E., Means, A. R., & Witters, L. A. (2005). The Ca^{2+}/calmodulin-dependent protein kinase kinases are AMPK-activated protein kinase kinases. *Journal of Biological Chemistry, 280*, 29060-29066.

Iglesias, M. A., Ye, J. M., Frangioudakis, G., Saha, A. K., Tomas, E., Ruderman, N. B., Cooney, G. J., & Kraegen, E. W. (2002). AICAR administration causes an apparent enhancement of muscle and liver insulin action in insulin-resistant high-fat-fed rats. *Diabetes, 51*, 2886-2894.

Ionescu, E., Sauter, J. F., & Jeanrenaud, B. (1985). Abnormal glucose tolerance in genetically obese (*fa/fa*) rats. *American Journal of Physiology, 248*, E500-E506.

Ip, C., Briggs, S. P., Haegele, A. D., Thompson, H. J., Storkson J. M., & Scimeca, J. A. (1996). The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet. *Carcinogenesis, 17*, 1045-1050.

Ip, C., Chin, S. F., Scimeca, J. A., & Pariza, M. W. (1991). Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. *Cancer Research, 51*, 6118-6124.

Invitti, C., Maffeis, C., Gilardini, L., Pontiggia, B., Mazzilli, G., Girol, A., Sartorio, A., Morabito., F., & Viberti, G. C. (2006). Metabolic syndrome in obese Caucasian children: prevalence using WHO-derived criteria and association with non-traditional risk factors. *International Journal of Obesity, 30*, 627-633.

Iwai, H., Ohno, Y., & Aoki, N. (2003). The effect of leptin, tumor necrosis factor-alpha (TNF-alpha), and nitric oxide (NO) production on insulin resistance in Otsuka Long-Evans fatty rats. *Endocrine Journal, 50*, 673-680.

Iwaki, M., Matsuda, M., Maeda, N., Funahashi T., Matsuzawa, Y., Makishima, M., & Shimomura, I. (2003). Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. *Diabetes, 52*, 1655-1663.

Fossati, P. & Lorenzo. P. (1982). Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clinical Chemistry, 28*, 2077-2080.

Kadowaki, T. & Yamauchi, T. (2005). Adiponectin and adiponectin receptors. *Endocrine Reviews, 26*, 439-451.

Katsuki, A., Sumida, Y., Gabazza, E. C., Murashima, S., Furuta, M., Araki-Sasaki, R., Hori, Y., Yano, Y., & Adachi, Y. (2001). Homeostasis model assessment is a reliable indicator of insulin resistance during follow-up of patients with Type 2 diabetes. *Diabetes Care, 24*, 362-365.

Katsuki, A., Sumida, Y., Murashima, S., Murata, K., Takarada, Y., Ito, K., Fujii, M., Tsuchihashi, K., Goto, H., Nakatani, K., & Yano, Y. (1998). Serum levels of tumor necrosis factor-α are increased in obese patients with noninsulin-dependent diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism, 83*, 859-862.

Kava, R., Greenwood, M. R. C., & Johnson, P. R. (1990). Zucker *fa/fa* rat. *Institute for Laboratory Animal Research Journal, 32* [Online]. Available: http://dels.nas.edu.ilnar_n/ilajournal/32_3/index.shtml.

Kava, R., Horowitz, C., Wojnar, Z., Turkenkopf, I., Johnson, P. R., & Greenwood, M. R. C. (1989). Short-term effects of adrenalectomy on adiposity, glycemia and glucose tolerance in obese and lean Zucker rats. *FASEB Journal, 3*, 356A.

Kazumi, T., Kawaguchi, A., Hirano, T., & Yoshino, G. (2004). Serum adiponectin is associated with high-density lipoprotein cholesterol, triglycerides, and low-density lipoprotein particle size in young healthy men. *Metabolism, 53*, 589-593.

Kelley, D. E., McKolanis, T. M., Hegazzi, R. A. F., Kuller, L. H., & Kalhan, S. C. (2003). Fatty liver in Type 2 diabetes mellitus: relation to regional adiposity, fatty acids, and insulin resistance. *American Journal of Physiology: Endocrinology and Metabolism, 285*, E906-E916.

Kepler, C. R. & Tove, S. B. (1967). Biohydrogenation of unsaturated fatty acids: III. Purification and properties of a linoleate delta-12-*cis*, delta-11-*trans*-isomerase from *Butyrivibrio fibrisolvens*. *Journal of Biological Chemistry, 242*, 5686-5692.

Kerouz, N. J., Horsch, D., Pons, S., & Kahn, C. R. (1997). Differential regulation of insulin receptor substrates-1 and –2 (IRS-1 and IRS-2) and phosphoinositol 3-kinase isoforms in liver and muscle of the obese diabetic (*ob/ob*) mouse. *Journal of Clinical Investigation, 100*, 3164-3172.

Kido, Y., Burks, D. J., Withers, D., Bruning, J. C., Kahn, C. R., White, M. F., & Accili, D. (2000). Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. *Journal of Clinical Investigation, 105,* 199-205.

Kim, H. K., Kim, S. R., Ahn, J. Y., Cho, I. J., Yoon, C. S., & Ha, T. Y. (2005). Dietary conjugated linoleic acid reduces lipid peroxidation by increasing oxidative stability in rats. *Journal of Nutritional Science and Vitaminology, 51*, 8-15.

Kloss, R., Linscheid, J., Johnson, A., Lawson, B., Edwards, K., Linder, T., Stocker, K., Petitte, J., & Kern, M. (2005). Effects of conjugated linoleic acid supplementation on blood lipids and adiposity of rats fed diets rich in saturated versus unsaturated fat. *Pharmacological Research, 51*, 503-507.

Krief, S. & Bazin, R. (1991). Genetic obesity: is the defect in the sympathetic nervous system? A review through developmental studies in the preobese Zucker rat. *Proceedings of the Society for Experimental Biology and Medicine, 198*, 528-538.

Krey, G., Braissant, O., L'Horset, F., Kalkoven, E., Perroud, M., Parker, M. G., & Wahli, W. (1997). Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator activated receptors by co-activator-dependent receptor ligand assay. *Molecular Endocrinology, 11*, 779-791.

Kruszynska, Y. T., Home, P. D., Hanning, I., & Alberti, K. G. (1987). Basal and 24-h C-peptide and insulin secretion rate in normal man. *Diabetologia, 30*, 16-21.

Kubota, N., Terauchi, Y., Yamamauci, T., Kubota, T., Moroi, M., Matsui, J., Eto, K., Yamashita, T., Kamon, J., Satoh, H., Yano, W., Froguel, P., Nagai, R., Kimura, S., Kadowaki, T., & Noda, T. (2002). Disruption of adiponectin causes insulin resistance and neointimal formation. *Journal of Biological Chemistry, 277*, 25863-25866.

Kurtz, T. W., Morris, R. C., & Pershadsingh, H. A. (1989). The Zucker fatty rat as a genetic model of obesity and hypertension. *Hypertension, 13*, 896-901.

Laasko, M. (1993). How good a marker is insulin level for insulin resistance? *American Journal of Epidemiology, 137*, 959-963.

Lang, C. H., Dobrescu, C., & Bagby, J. (1992). Tumor necrosis factor impairs insulin action on peripheral glucose disposal and hepatic glucose output. *Endocrinology, 130*, 43-52.

Lauterio, T. J., Bond, J. P., & Ulman, E. A. (1994). Development and characterization of purified diet to indentify obesity-susceptible and resistant rat populations. *Journal of Nutrition, 124*, 2172-2178.

Lee, J. H., Cho, K. H., Lee, K. T., & Kim, M. R. (2005). Antiatherogenic effects of structured lipid containing conjugated linoleic acid in C57BL/6J mice. *Journal of Agricultural and Food Chemistry, 53*, 7295-7301.

Lee, W. J., Kim, M., Park, H. S., Kim, H. S., Jeon, M. J., Oh, K. S., Won, J. C., Kim, M. S., Oh, G. T., Yoon, M., Lee, K. U., & Park, J. Y. (2006). AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARalpha and PGC-1. *Biochemical and Biophysical Research Communications, 340*, 291-295.

Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkinson, W. O., Willson, T. M., & Kliewer, S. A. (1995). An antidiabetic thiazoledinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *Journal of Biological Chemistry, 270*, 12953-12956.

Li, Z., Yang, S., Lin, H., Huang, J., Watkins, PA., Moser, AB., Desimone, C., Song, X. Y., & Diehl, A. M. (2003). Probiotics and antibodies to TNF-α inhibit inflammatory activity and improve nonalcoholic fatty liver disease. *Hepatology, 37*, 343-350.

Liang, G., Yang, J., Horton, J. D., Hammer, R. E., Goldstein, J. L., & Brown, M. S. (2002). Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. *Journal of Biological Chemistry, 277*, 9520-9528.

Lizcano, J. M. & Alessi, D. R. (2002). The insulin signalling pathway. *Current Biology, 12*, R236-R238.

Luo, Y., Corning, B. F., Fisher, T. F., Morin, R. R., Andrews, D. R., & Owens, D. R. (2006). Evaluation of portable glucometers for use in rats [On-line]. Available: http://www.geneticmodels.com/flex_content_area/documents/rm_dm_r_glucometer_evaluation_presentation.pdf.

Lui, J., Hanley, A. J. G., Young, T. K., Harris, S. B. & Zinman, B. (2005). Characteristics and prevalence of the metabolic syndrome among three ethnic groups in Canada. *International Journal of Obesity, 30*, 669-676.

Liu, J., Young, T. K., Zinman, B., Harris, S. B., Connelly, P. W., & Hanley, A. J. (2006). Lifestyle variables, non-traditional cardiovascular risk factors, and the metabolic syndrome in an Aboriginal Canadian population. *Obesity, 14*, 500-508.

Lochhead, P. A., Salt, I.P., Walker, K. S., Hardie, D. G., & Sutherland, C. (2000). 5-aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase. *Diabetes, 49*, 896-903.

Long, Y. C. & Zierath, J. R. (2006). AMP-activated protein kinase signaling in metabolic regulation. *Journal of Clinical Investigation, 116*, 1776-1782.

Luyckx, F. H., Lefebvre, P. J., & Scheen, A. J. (2000). Non-alcoholic steatohepatitis: association with obesity and insulin resistance, and influence of weight loss. *Diabetes & Metabolism, 26*, 98-106.

Maeda, K., Ishihara, K., Miyake, K., Kaji, Y., Kawamitsu, H., Fujii, M., Sugimura, K., & Ohara, T. (2005). Inverse correlation between serum adiponectin concentration and hepatic lipid content in Japanese with Type 2 diabetes. *Metabolism, 54*, 775-780.

Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Matsuda, M., Nagaretani, H., Furuyama, N., Kondo, H., Takahashi, M., Arita, Y., Komura, R., Ouchi, N., Kihara, S., Tochino, Y., Okutomi, K., Horie, M., Takeda, S., Aoyama, T., Funahashi, T., & Matsuzawa, Y. (2002). Diet-induced insulin resistance in mice lacking adiponectin/ACRP-30. *Nature Medicine, 8*, 731-737.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R. E., Lee, G. H., Zhang, Y., Fei, H., Kim, S., Lallone, R., & Ranganathan, S. (1995). Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nature Medicine, 1*, 1155-1161.

Malnick, S. D. H., Beergable, M., & Knobler, H. (2003). Non-alcoholic fatty liver: a common manifestation of a metabolic disorder. *QJM-An International Journal of Medicine, 96*, 699-709.

Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Bugianesi, E., Lenzi, M., McCullough, A. J., Natale, S., Forlani, G., & Melchionda, N. (2001). Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes, 50*, 1844-1850.

Marchesini, G., Brizi, M., Morselli-Labate, A. M., Biachi, G., Buigianesi, E., McCullough, A. J., Forlani, G., & Melchionda, N. (1999). Association of non-alcoholic fatty liver disease with insulin resistance. *American Journal of Medicine, 107*, 450-455.

Marques, R. G., Fontaine, M. J., & Rogers, J. (2004). C-peptide: much more than a byproduct of insulin biosynthesis. *Pancreas, 29*, 231-238.

Matsubara, M., Maruoka, S., & Katayose, S. (2002). Decreased plasma adiponectin concentrations in women with dyslipidemia. *Journal of Clinical Endocrinology and Metabolism, 87*, 2764-2769.

McCullough, A. J. (2004). The clinical features, diagnosis, and natural history of non-alcoholic fatty liver disease. *Clinics in Liver Disease, 8*, 521-533.

McGarry, J. D. & Brown, N. F. (1997). The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *European Journal of Biochemistry, 244*, 1-14.

McGowan, M. W., Artiss, J. D., Strandberg, D. R., & Zak, B. (1983). A peroxidase coupled method for the colorimetric determination of serum triglycerides. *Clinical Chemistry, 29*, 538-542.

McTernan, P. G., Kusminski, C. M., & Kumar, S. (2006). Resistin. *Current Opinion in Lipidology, 17*, 170-175.

Mehta, K., Van Thiel, D. H., Shah, N., & Mobarham, S. (2002). Nonalcoholic fatty liver disease: pathogenesis and the role of antioxidants. *Nutrition Reviews, 60*, 289-293.

Mercer, J. G. & Archer, Z. A. (2005). Diet-induced obesity in the Sprague-Dawley rat: dietary manipulations and their effect on hypothalamic neuropeptide energy balance systems. *Biochemical Society Transactions, 33*, 1068-1072.

Mokdad, A. J., Ford, E. S., Bowman, B. A., Dietz, W. H., Vinicor, F., Bales, V. S., & Marks, J. S. (2003). Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *Journal of the American Medical Association, 289*, 76-79.

Moloney, F., Yeow, T. P., Mullen, A., Nolan, J. J., & Roche, H. M. (2004). Conjugated linoleic acid supplementation, insulin sensitivity, and lipoprotein metabolism in patients with Type 2 diabetes mellitus. *American Journal of Clinical Nutrition, 80*, 887-895.

Moya-Camarena, S. Y., Vanden Heuvel, J. P., Blanchard, S. G., Leesnitzer, L. A., & Belury, M. A. (1999). Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARα. *Journal of Lipid Research, 40*, 1426-1433.

Muoio, D. M., Seefeld, K., Witters, L. A., & Coleman R. A. (1999). AMP-activated protein kinase reciprocally refulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that *sn*-glycerol-3-phosphate acyltransferase is a novel target. *Biochemical Journal, 338*, 783-791.

Muse, E. D., Obici, S., Bhanot, S., Monia, B. P., McKay, R. A., Rajala, M. W., Scherer, P. E., & Rossetti, L. Role of resistin in diet-induced hepatic insulin resistance. *Journal of Clinical Investigation, 114*, 232-239.

Nagao, K., Inoue, N., Wang, Y. M., Shirouchi, B., & Yanagita, T. (2005). Dietary conjugated linoleic acid alleviates nonalcoholic fatty liver disease in Zucker (*fa/fa*) rats. *Journal of Nutrition, 135*, 9-13.

Nagao, K., Inoue, N., Wang, Y. M., Hirata, J., Shimada, Y., Nagao, T., Matsui, T., & Yanagita, T. (2003a). The 10*trans*, 12*cis* isomer of conjugated linoleic acid suppresses the development of hypertension in Otsuka Long-Evans Tokushima fatty rats. *Biochemical and Biophysical Research Communications, 306*, 134-138.

Nagao, K., Inoue, N., Wang, Y.-M., & Yanagita, T. (2003b). Conjugated linoleic acid enhances plasma adiponectin level and alleviates hyperinsulinemia and hypertension in Zucker diabetic fatty (*fa/fa*) rats. *Biochemical and Biophysical Research Communications, 310*, 562-566.

Nagao, K., Wang, Y.-M., Inoue, N., Han, S.-Y., Buang, Y., Noda, T., Kouda, N., Okamatsu, H., & Yanagita, T. (2003c). The 10trans, 12cis isomer of conjugated linoleic acid promotes energy metabolism in OLETF rats. *Nutrition, 19*, 652-656.

Nair, S., Diehl, A. M., Wiseman, M., Farr, G. H. Jr., & Perrillo, R. P. (2004). Metformin in the treatment of non-alcoholic steatohepatitis: a pilot open label trial. *Alimentary Pharmacology & Therapy, 20*, 23-28.

National Cholesterol Education Program. (2001). Executive summary of the third report of the national cholesterol education program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). *Journal of the American Medical Association, 285*, 2486-2497.

Neuschwander-Tetri, B. A. & Caldwell, S. M. (2003). Non-alcoholic steatohepatits: Summary of an AASLD single topic conference. *Hepatology, 37*, 1202-1219.

Noto, A. (2004). Conjugated linoleic acid (CLA) in liver and adipose metabolism: Effects on insulin resistance, adiposity, hepatic steatosis and lipidemia [dissertation]. Winnipeg (Manitoba): University of Manitoba.

Noto, A., Zahradka, P., Yurkova, N., Xie, X., Nitschmann, E., Ogborn, M., & Taylor, C. G. (2006). Conjugated linoleic acid (CLA) reduces hepatic steatosis, improves liver function, and favorably modifies lipid metabolism in obese insulin resistant rats. *Lipids, 41*, 179-188.

Oana, F., Takeda, H., Hayakawa, K., Matsuzawa, A., Akahane, S., Isaji, M., & Akahane, M. (2005). Physiological difference between obese (*fa/fa*) Zucker rats and lean Zucker rats concerning adiponectin. *Metabolism Clinical and Experimental, 54*, 995-1001.

Office of Nutrition Policy and Promotion. (2003). Canadian guidelines for body weight classification in adults. Health Canada: Ottawa, Canada.

Olfert, E., Cross, B., & McWilliam, A. (1993). Guide to the Care and Use of Experimental Animals.

Oliver, W. R. Jr., Shenk, J. L., Snaith, M. R., Russell, C. S., Plunket, K. D., Bodkin, N. L., Lewis, M. C., Winegar, D. A., Sznaidman, M. L., Lambert, M. H., Xu, H. E., Sternbach, D. D., Kliewer, S. A., Hansen, B. C., & Willson, T. M. (2001). A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. *Proceedings of the National Academy of Science, 98*, 5306-5311.

Pagano, C., Soardo, G., Esposito, W., Fallo, F., Basan, L., Donnini, D., Federspil, G., Sechi, L. A., & Vettor, R. (2005). Plasma adiponectin is decreased in nonalcoholic fatty liver disease. *European Journal of Endocrinology, 152*, 113-118.

Park., Y., Albright, K. J., Liu, W., Cook, M. E., & Pariza, M.W. (1997). Effect of conjugated linoleic acid on body composition in mice. *Lipids, 32*, 853-858.

Pariza, M., Park, Y., & Cook, M. (2001). The biologically active isomers of conjugated linoleic acid. *Progress in Lipid Research, 40*, 283-298.

Perez-Carreras, M., Del Hoyo, P., Martin, M. A., Rubio, J. C., Martin, A., Castellano, G., Colina, F., Arenas, J., & Solis-Herruzo, J. A. (2003). Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology, 38*, 999-1007.

Peters, J. M., Park, Y., Gonzalez, F. J., & Pariza, M. W. (2001). Influence of conjugated linoleic acid on body composition and target gene expression in peroxisome proliferator-activated receptor alpha-null mice. *Biochimica et Biophysica Acta, 1533*, 233-242.

Peterson, D. G., Matitashvili, E. A., & Bauman, D. E. (2004). The inhibitory effect of trans-10, cis-12 CLA on lipid synthesis in bovine mammary epithelial cells involves reduced proteolytic activation of the transcription factor SREBP-1. *Journal of Nutrition, 134*, 2523-2527.

Peterson, R. G., Shaw, W. N., Neel, M.-A., Little, L. A., & Eichberg, J. (1990). Zucker diabetic fatty rat as a model for non-insulin-dependent diabetes mellitus. *Institute for Laboratory Animal Research Journal, 32* [Online]. Retrieved May 3, 2006, from http://dels.nas.edu.ilnar_n/ilajournal/32_3/index.shtml.

Phillips, M. S., Lui, Q., Hammond, H. A., Dugan, V., Hey, P. J., Caskey, C. J., & Hess, J. F. (1996). Leptin receptor missense mutation in the fatty Zucker rat. *Nature Genetics, 13*, 18-19.

Pierce, L. C. (2003). Metabolic syndrome & obesity: co-epidemics could overwhelm home health care. *Caring, 22*, 24-28.

Pirola, L., Johnston, A. M., & Van Obberghen, E. (2004). Modulation of insulin action. *Diabetologia, 47*, 170-184.

Previs, S. F., Withers, D. J., Ren, J. M., White, M. F., & Shulman, G. I. (2000). Contrasting effects of IRS-1 versus IRS-2 gene disruption on carbohydrate and lipid metabolism in vivo. *Journal of Biological Chemistry, 275*, 38990-38994.

Promrat, K., Lutchman, G., Uwaifo, GI., Freedman, R. J., Soza, A., Heller, T., Doo, E., Ghany, M., Premkumar, A., Park, Y., Liang, T.J., Yanovski, J. A., Kleiner, D. E., & Hoofnagle, J. H. (2004). A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. *Hepatology, 39*, 188-196.

Rahman, S. M., Wang, Y.-M., Yotsumoto, H., Cha, J.-Y., Han, S.-Y., Inoue, S., & Yanagita, H. (2001). Effects of conjugated linoleic acid on serum leptin concentration, body-fat accumulation, and β-oxidation of fatty acid in OLETF rats. *Nutrition, 17*, 385-390.

Rajala, M. W., Obici, S., Scherer, P. E., & Rosetti, L. (2003). Adipose-derived resistin and gut-derived resistin-like molecul-beta selectively impair insulin action on glucose production. *Journal of Clinical Investigation, 114*, 232-239.

Reaven, G. M. (1988). Banting lecture. Role of insulin resistance in human disease. *Diabetes, 37*, 15-95-1607.

Reeves, P. G., Nielsen, F. H., & Fahey, C. G., Jr. (1993). AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *Journal of Nutrition, 123*, 1939-1951.

Ren, B., Thelen, A. P., Peters, J. M., Gonzalez, F. J., & Jump, D. B. (1997). Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor α. *Journal of Biological Chemistry, 272*, 26827-26832.

Ridker, P. M., Wilson, P. W. F., & Grundy, S. M. (2004). Should C-Reactive Protein Be Added to Metabolic Syndrome and to Assessment of Global Cardiovascular Risk? *Circulation, 109*, 2818-2825.

Rieusset, J., Bouzakri, K., Chevillotte, E., Richard, N., Jacquet, D., Bastard, J. P., Laville, M., & Vidal, H. (2004). Suppressor of cytokine signaling 3 expression and insulin resistance in skeletal muscle of obese and type 2 diabetic patients. *Diabetes, 53*, 2232-2241.

Risérus, U., Arner, P., Brismar, K., & Vessby, B. (2002a). Treatment with dietary trans10,cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. *Diabetes Care, 25*, 840-847.

Risérus, U., Basu, S., Jovinge, S., Fredrikson, G. N., Arnlov, J., & Vessby, B. (2002b). Supplementation with conjugated linoleic acid causes isomer-dependent oxidative stress and elevated C-reactive protein: a potential link to fatty acid-induced insulin resistance. *Circulation, 106*, 1925-1929.

Risérus, U, Berglud, L., & Vessby, B. (2001). Conjugated linoleic acid (CLA) reduced abdominal adipose tissue in obese middle-aged men with signs of the metabolic syndrome: a randomised controlled trial. *International Journal of Obesity and Related Metabolic Disorders, 25*, 1129-1135.

Roberts, M. J. (1999). M. J. Roberts & Russo R. (Eds.), *A Student's Guide to Analysis of Variance*. New York, NY: Routledge.

Roche, H. M., Noone, E., Sewter, C., Mc Bennett S., Savage, D., Gibney, M. J., O'Rahilly, S., & Vidal-Puig, A. J. (2002). Isomer-dependent metabolic effects of conjugated linoleic acid: insights from molecular markers sterol regulatory element-binding protein-1c and LXRalpha. *Diabetes, 51*, 2037-2044.

Roschlau, P., Bernt, E., & Gruber, W. (1974). Enzymatishe bestimmung des cesamt-cholesterins in serum. *Z. Klin. Chem. Klin. Biochem. 12*, 226.

Rother, K. I., Imai, Y., Caruso, M., Beguino, F., Formisano, P., & Accili, D. (1998). Evidence that IRS-2 phosphorylation is required for insulin action in hepatocytes. *Journal of Biological Chemistry, 273*, 17491-17497.

Ruderman, N. B., Saha, A. K., & Kraegen, E. W. (2003). Minireview: malonyl CoA, AMP-activated protein kinase, and adiposity. *Endocrinology, 144*, 5166-5171.

Ruderman, N. B., Saha, A. K., Vavvas, D., & Witters, L.A. (1999). Malonyl-CoA, fuel sensing, and insulin resistance. *American Journal of Physiology, 276*, E1-E18.

Rui, L., Aguirre, V., Kim, J. K., Shulman, G. I., Lee, A., Corbould, A., Dunaif, A., & White, M. F. (2001). Insulin/IGF-1 and TNF-alpha stimulate phosphorylation of IRS-1 at inhibitory Ser307 via distinct pathways. *Journal of Clinical Investigation, 107*, 181-189.

Russell, J. C. (1995). The atherosclerosis-prone JCR:LA-corpulent rat. In F. P. Woodford, J. Davignon, & A. Sniderman (eds.) Atherosclerosis X: Proceedings of the 10th International Symposium on Atherosclerosis (pp. 121-125). Amsterdam: Elselvier.

Russell, J. C. & Koeslag, D. G. (1990). Jcr:LA-corpulent rat: a strain with spontaneous vascular and myocardial disease. *Institute for Laboratory Animal Research Journal, 32* [Online].

Ryder, J. W., Portocarrero, C. P., Song, X. M., Cui, L., Yu, M., Combatsiaris, T., Galuska, D., Bauman, D. E., Barbano, D. M., Charron, M. J., Zierath, J. R., & Houseknecht, K. L. (2001). Isomer-specific antidiabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. *Diabetes, 50*, 1149-1157.

Sakai, J. & Rawson, R. B. (2001). The sterol regulatory element binding protein pathway: control of lipid homeostasis through regulated intracellular transport. *Current Opinion in Lipidology, 12*, 261-266.

Salas-Salvadó, J., Márquez-Sandoval, F., & Bulló, M. (2006). Conjugated linoleic acid intake in humans: a systemic review focusing on its effects on body composition, glucose, and lipid metabolism. *Critical Reviews in Food Science and Nutrition, 46*, 479-488.

Saltiel, A. R. & Kahn, R. C. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature, 414*, 799-806.

Samuel, V. T., Liu, Z.-X., Qu, X., Elder, B. D., Bilz, S., Befroy, D., Romanelli, A. J., & Shulman, G. I. (2004). Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *Journal of Biological Chemistry, 279*, 32345-32353.

Sanders, S. R., Teachey, M. K., Ptock, A., Kraemer, K., Hasselwander, O., Henriksen, E. J., & Baumgard, L. H. (2004). Effects of specific conjugated linoleic acid isomers on growth characteristics in obese Zucker rats. *Lipids, 39*, 537-543.

Santora, J. E., Palmquist, D. L., & Roehrig, K. L. (2000). *Trans*-vaccenic acid is desaturated to conjugated linoleic acid in mice. *Journal of Nutrition, 130*, 208–215.

Sanyal, A. J., Mofrad, P. S., Contos, M. J., Sargeant, C., Luketic, V. A., Sterling, R. K., Stravitz, R. T., Shiffman, M. L., Clore, J., & Mills, A. S. (2004). A pilot study of vitamin E versus vitamin E and pioglitazone for the treatment of nonalcoholic steatohepatitis. *Clinical Gastroenterology and Hepatology, 2*, 1107-1115.

Sarafidis, P. A. & Nilsson, P. M. (2006). The metabolic syndrome: a glance at its history. *Journal of Hypertension, 24*, 621-626.

Schindhelm, R. K., Diamant, M., Dekker, J. M., Tushuizen, M. E., Teerlink, T., & Heine, R. J. (2006). Alanine aminotransferase as a marker of non-alcoholic fatty liver disease in relation to Type 2 diabetes mellitus and cardiovascular disease. *Diabetes/Metabolism Research Reviews,* July 11, 2006 (E-pub ahead of print).

Sekiya, M., Yahagi, N., Matsuzaka, T., Najima, Y., Nakakuki, M., Nagai, R., Ishibashi, S., Osuga, J., Yamada, N., & Shimano, H. (2003). Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology, 38*, 1529-1539.

Senn, J. J., Klover, P. J., Nowak, I. A., Zimmers, T. A., Koniaris, L. G., Furlanetto, R. W., & Mooney, R. A. (2003). Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. *Journal of Biological Chemistry, 278*, 13740-13746.

Seppala-Lindroos, A., Hakkinen, A. M., Goto, T., Westerbacka, J., Sovijarvi, A., Halavaara, J., & Yki-Jarvinen, H. (2002). Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum fatty acids independent of obesity in normal men. *Journal of Clinical Endocrinology and Metabolism, 87*, 2206-2211.

Shand, B. I., Scott, R. S., Elder, P. A., & George, P. M. (2003). Plasma adiponectin in overweight, nondiabetic individuals with or without insulin resistance. *Diabetes Obesity and Metabolism, 5*, 349-353.

Sheth, S. G., Gordon, F.D., & Chopra, S. (1997). Non-alcoholic steatohepatitis. *Annals of Internal Medicine, 126*, 137-145.

Smedman, A. &Vessby, B. (2001). Conjugated linoleic acid supplementation in humans – metabolic effects. *Lipids, 36*, 773-781.

Shimano, H., Horton, J. D., Hammer, R. E., Shimomura, I., Brown, M. S., & Goldstein, J. L. (1996). Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *Journal of Clinical Investigation, 98*, 1575-1584.

Shimano, H., Horton, J. D., Shimomura, I., Hammer, R. E., Brown, M. S., & Goldstein, J. L. (1997). Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *Journal of Clinical Investigation, 99*, 846-854.

Shimano, H., Yahagi, N., Amemiya-Kudo, M., Hasty, A. H., Osuga, J., Tamura, Y., Shionoiri, F., Iizuka, Y., Ohashi, K., Harada, K., Gotoda, T, Ishibashi, S., & Yamada, N. (1999). Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. *Journal of Biological Chemistry, 274*, 35832-35839.

Shimomura, I., Matsuda, M., Hammer, R. E., Bashmakov, Y., Brown, M. S., & Goldstein, J. L. (2000). Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and *ob/ob* mice. *Molecular Cell, 6*, 77-86.

Shulman, G. I. (1999). Cellular mechanisms of insulin resistance in humans. *American Journal of Cardiology, 84*, 3J-10J.

Silha, J. V., Krsek, M., Skrha, J. V., Sucharda, P., Nyomba, B. L., & Murphy, L. J. (2003). Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. *European Journal of Endocrinology, 149*, 331-335.

Sisk, M. B., Hausman, D. B., Martin, R. J., & Azain, M. J. (2001). Dietary conjugated linoleic acid reduces adiposity in lean but not obese Zucker rats. *Journal of Nutrition, 131*, 1668-1674.

Smedman, A., Basu, S., Jovinge, S., Fredrikson, G. N., & Vessby, B. (2005). Conjugated linoleic acid increased C-reactive protein in human subjects. *British Journal of Nutrition, 94*, 791-795.

Smith, S. A. (2003). Central role of the adipocyte in the insulin-sensitizing and cardiovascular risk modifying actions of the thiazoledinediones. *Biochime, 85*, 1219-1230.

Smith, E. M., Finn, S. G., Tee, A. R., Browne, G. J., & Proud, C. G. (2005). The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. *Journal of Biological Chemistry, 280*, 18717-18727.

Spiegelman, B. M. & Hotamisligil, G. S. (1993). Through thick and thin: wasting, obesity, and TNF alpha. *Cell, 73*, 625-627.

Steppan, C. M., Wang, J., Whiteman, E. L., Birnbaum, M. J., & Lazar, M. A. (2005). Activation of SOCS-3 by resistin. *Molecular and Cellular Biology, 25*, 1569-1575.

Stone, B. G. & Van Thiel, D. H. (1985). Diabetes mellitus and the liver. *Seminars in Liver Disease, 5*, 8-28.

Suchankova, G., Tekle, M., Saha, A. K., Ruderman, N. B., Clarke, S. D., & Gettys, T. W. (2005). Dietary polyunsaturated fatty acids enhance hepatic AMP-activated protein kinase activity in rats. *Biochemical and Biophysical Research Communications, 326*, 851-858.

Sugano, M., Tsujita, A., Yamasaki, M., Noguchi, M., & Yamada, K. (1998). Conjugated linoleic acid modulates tissue levels of chemical mediators and immunoglobulins in rats. *Lipids, 33*, 521-527.

Takahashi, Y., Kushiro, M., Shinohara, K., & Ide, T. (2003). Activity and mRNA levels of enzymes involved in hepatic fatty acid synthesis and oxidation in mice fed conjugated linoleic acid. *Biochimica et Biophysica Acta, 1631*, 265-73.

Teachey, M. K., Taylor, Z. C., Maier, T., Saengsirisuwan, V., Sloniger, J. A., Jacob, S., Klatt, M. J., Ptock, A., Kraemer, K., Hasselwander, O., & Henrickson, E. J. (2003). Interactions of conjugated linoleic acid and lipoic acid on insulin action in the obese Zucker rat. *Metabolism, 52*, 1167-1174.

Terrettaz, J., Assimacopoulos-Jeannet, F., & Jeanrenaud, B. (1986a). Inhibition of hepatic glucose production by insulin in vivo in rats: Contribution of glycolysis. *American Journal of Physiology, 250*, E346-E351.

Terrettaz, J., Assimacopoulos-Jeannet, F., & Jeanrenaud, B. (1986b). Severe hepatic and peripheral insulin resistance as evidenced by euglycemic clamps in genetically obese *fa/fa* rats. *Endocrinology, 118*, 674-678.

Tilg, H. & Diehl, A. M. (2000). Cytokines in alcoholic and non-alcoholic steatohepatitis. *New England Journal of Medicine, 343*, 1467-1476.

Tjepkema, M. (2005). Adult obesity in Canada: measured height and weight. Nutrition: Findings from the Canadian Community Health Survey [On-line]. Available: http://www.statcan.ca/english/research/82-620-MIE/2005001/articles/adults/aobesity.htm.

Tricon, S., Burdge, G., Kew, S., Banerjee, T., Tussell, J., Jones, E., Grimble, R., Williams, C., Yaqoob, P., & Calder, P. (2004). Opposing effects of cis-9, trans-11 and trans-10, cis-12 conjugated linoleic acid on blood lipids in healthy humans. *American Journal of Clinical Nutrition, 80*, 614-620.

Trinder, P. (1969). Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. *Journal of Clinical Pathology 22*, 158-161.

Tsao, T. S., Lodish, H. F., & Fruebis, J. (2002). ACRP30, a new hormone controlling fat and glucose metabolism. *European Journal of Pharmacology, 440*, 213-221.

Tsuboyama-Kasaoka, N., Takahashi, M., Tanemura, K., Kim, H.-J., Tange, T., Okuyama, H., Kasai, M., Ikemoto, S., & Ezaki, O. (2000). Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes, 49*, 1534-1542.

Turpeinen, A. M., Mutanen, M., Aro, A., Salminen, I., Basu, S., Palmquist, D. L., & Griinari, J. M. (2002). Bioconversion of vaccenic acid to conjugated linoleic acid in humans. *American Journal of Clinical Nutrition, 76*, 504–510.

Uygun, A., Kadayifci, A., Isik, A. T., Ozgurtas, T., Deveci, S., Tuzun, A., Yesilova, Z., Gulsen, M., & Dagalp, K. (2004). Metformin in the treatment of patients with non-alcoholic steatohepatitis. *Alimentary Pharmacology & Therapeutics, 19*, 537-544.

Valverde, A. M., Burks, D. J., Fabregat, I., Fisher, T. L., Carretero, J., White, M. F., & Benito, M. (2003). Molecular mechanisms of insulin resistance in IRS-2 deficient hepatocytes. *Diabetes, 52*, 2239-2248.

Van Steenbergen, W. & Lanckmans, S. (1995). Liver disturbances in obesity and diabetes mellitus. *International Journal of Obesity and Related Metabolic Disorders, 19*(Suppl. 3), S27-S36.

Vos Louthan, M., Barve, S., McClain, C. J., & Joshi-Barve, S. (2005). Decreased serum adiponectin: an early event in pediatric nonalcoholic fatty liver disease. *Journal of Pediatrics, 147*, 835-838.

Wahli, W. (2002). Peroxisome proliferator-activated receptors (PPARs): From metabolic control to epidermal wound healing. *Swiss Medical Weekly, 132*, 83-91.

Wallace, T. M., Levy, J. C., & Matthews, D. R. (2004). Use and abuse of HOMA modeling. *Diabetes Care, 27*, 1487-1495.

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D. R., & Proud, C. G.. (2001). Regulation of elongation factor 2 kinase by p90RSK1 and p70 S6 kinase. *EMBO Journal, 20*, 4370-4379.

Wang, Y.-M., Nagao, K., Inoue, N., Ujino, Y., Shimada, Y., Nagao, T., Iwata, T., Kamegai, T., Yamaguchi-Sato, Y., & Yanagita, T. (2006). Isomer-specific anti-obese and and hypolipidemic properties of conjugated linoleic acid in obese OLETF rats. *Bioscience, Biotechnology, and Biochemistry, 70*, 355-360.

Wanless, I. R. & Lentz, J. S. (1990). Fatty liver hepatitis (steatohepatits) and obesity: An autopsy study with analysis of risk factors. *Hepatology, 12*, 1106-1110.

Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., & Ferrante, A. W. Jr. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigation, 112*, 1796-1808.

Westerbacka, J., Corner, A., & Tiikkainen, M. (2004). Women and men have similar amounts of liver and intra-abdominal fat, despite more subcutaneous fat in women: implications for sex differences in markers of cardiovascular risk. *Diabetologia, 47*, 1360-1369.

Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R. E., & Tataranni, P. A. (2001). Hypoadiponectinemia in obesity and Type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *Journal of Clinical Endocrinology and Metabolism, 86*, 1930-1935.

Whigham, L., O;Shea, M., Mohede, I., Walaski, H., & Atkinson, R. (2004). Safety profile of conjugated linoleic acid in a 12-month trial in obese humans. *Food Chemistry and Toxicology, 42*, 1701-1709.

White, M. F. (2002). IRS proteins and the common path to diabetes. *American Journal of Physiology: Endocrinology, and Metabolism, 283*, E413-E422.

White, D. W., Wang, D. W., Chua, S. C. Jr, Morgenstern, J. P., Leibel, R. L., Baumann, H., & Tartaglia, L. A. (1997). Constitutive and impaired signaling of leptin receptors containing the Gln→Pro extracellular domain *fatty* mutation. *Proceedings of the National Academy of Science U. S. A., 94*, 10657-10662.

Wilson, T. M., Cobb, J. E., Cowan, D. J., Wiethe, R. W., Correa, I. D., Prakash, S. R., Beck, K. D., Moore, L. B., Kliewer, S. A., & Lehmann, J. M. (1996). The structure-activity relationship between peroxisome proliferator-activated receptor γ agonism and the antihyperglycemic activity of thiazoledinediones. *Journal of Medicinal Chemistry, 39*, 665-668.

Wilson, T. A., Nicolosi, R. J., Chrysam, M., & Kritchevsky, D. (2000). Conjugated linoleic acid reduces early aortic atherosclerosis greater than linoleic acid in hypercholesterolemic hamsters. *Nutrition Research, 17*, 987-994.

Wilson, T. A., Nicolosi, R. J., Saati, A., Kotyla, T., & Kritchevsky, D. (2006). Conjugated linoleic acid isomers reduce blood cholesterol levels but not aortic cholesterol accumulation in hypercholesterolemic hamsters. *Lipids, 41*, 41-48.

Winder, W. W. & Hardie, D. G. (1999). AMP-activate protein kinase, a metabolic master switch: possible roles in Type 2 diabetes. *American Journal of Physiology, 277*, E1-E10.

Woods, A., Azzout-Marniche, A., Foretz, M., Stein, S. C., Lemarchand, P., Ferré, P., Foufelle, F., & Carling, D. (2000). Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. *Molecular and Cellular Biology, 20*, 6704-6711.

Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G. D., Neuman, D., Schlattner, U., Walliman, T., Carlson, M., & Carling, D. (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Current Biology*, *13*, 2004-2008.

World Health Organization. (1999). Obesity and overweight [On-line]. Available: http://www.who.int/dietphysicalactivity/publications/facts/obesity/en/.

Xu, J., Nakamura, M. T., Cho, H. P., & Clarke, S.D. (1999). Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. *Journal of Biological Chemistry, 274*, 23577-23583.

Xu, J., Teran-Garcia, M., Park, J. H. Y., Nakamura, M. T., & Clarke, S. D. (2001). Polyunsaturated fatty acids suppress hepatic sterol regulatory element-binding protein-1 expression by accelerating transcript delay. *Journal of Biological Chemistry, 276*, 9800-9807.

Xu, A., Wang, Y., Keshaw, H., Xu, L. Y., Lam, K. S., & Cooper, G. J. (2003). The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. *Journal of Clinical Investigation, 112*, 91-100.

Yahagi, N., Shimano, H., Hasty, A. H., Matsuzaka, T., Ide, T., Yoshikawa, T., Amemiya-Kudo, M., Tomita, S., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., & Yamada, N. (2002). Absence of sterol regulatory element-binding protein-1 (SREBP-1) ameliorates fatty livers but not obesity or insulin resistance in Lep^{ob}/Lep^{ob} mice. *Journal of Biological Chemistry, 277*, 19353-19357.

Yamasaki, M., Ikeda, A., Oji, M., Tanaka, Y., Hirao, A., Kasai, M., Iwata, T., Tachibana, H., & Yamada, K. (2003). Modulation of body fat and serum leptin levels by dietary conjugated linoleic acid in Sprague Dawley rats fed various fat-level diets. *Nutrition, 19*, 30-35.

Yamasaki, M., Mansho, K., Ogino, Y., Kasai, M., Tachibana, H., & Yamada, K. (2000). Acute reduction of serum leptin level by dietary conjugated linoleic acid in Sprague Dawley rats. *Journal of Nutritional Biochemistry, 11*, 467-471.

Yamashita, T., Murakami, T., Iida, M., Kuwajima, M., & Shima, K. (1997). Leptin receptor of Zucker fatty rat performs reduced signal transduction. *Diabetes, 46*, 1077-1080.

Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., Ezaki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M. L., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Tobe, K., Nagai, R., Kimura, S., Tomita, M., Froguel, P., & Kadowaki, T. (2001). The fat-derived hormone adiponectin reverses insulin resistance associated with both lipodystrophy and obesity. *Nature Medicine, 7*, 941-946.

Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., & Kadowaki, T. (2002). Adiponectin stimulates utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature Medicine, 8*, 1288-1295.

Yang, A. (1999). Delta (9) desaturase activity in bovine subcutaneous adipose tissue of different fatty acid composition. *Lipids, 34*, 971-978.

Yang, W. S., Lee, W. J., Funahashi, T., Tanaka, S., Matsuzawa, Y., Chao, C. L., Chen, C. L., Tai, T. Y., & Chuang, L. M. (2002). Plasma adiponectin levels in overweight and obese Asians. *Obesity Research, 10*, 1104-1110.

Yoon, D., Lee, S. H., Park, H. S., Lee, J. H., Park, J. S., Cho, K. H., & Kim, S. M. (2005). Hypoadiponectinemia and insulin resistance are associated with non-alcoholic fatty liver disease. *Journal of Korean Medical Science, 20*, 421-426.

Yoshikawa, T., Shimano, H., Yahagi, N., Ide, T., Amemiya-Kudo, M., Matsuzaka, T., Nakakuki, M., Tomita, S., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Takahashi, A., Sone, H., Osuga, J., Gotoda, T., Ishibashi, S., & Yamada, N. (2002). Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *Journal of Biological Chemistry, 277*, 1705-1711.

Younossi, Z. M., Gramlich, T., Bacon, B. R., Matteoni, C. A., Bopari, N., O'Neill, R., & McCullough, A. J. (1999). Hepatic iron and non-alcoholic fatty liver disease. *Hepatology, 30,* 847-850.

Yurkova, N., Xie, X., Ryz, N., Defries, D., Herchak, D., Diakiw, R., Zahradka, J., Lee, L., Zahradka, P., & Taylor, C.G. (2004). Regulation of resistin expression in adipose tissue by conjugated linoleic acid (CLA) isomers in *fa/fa* Zucker rats. *Diabetes 53*: A355.

Zavaroni, I., Deferrari, G., Lugari, R., Bonora, E., Garibotto, G., Dall'Aglio, E., Robaudo, C., & Gnudi, A. (1987). Renal metabolism of C-peptide in man. *Journal of Clinical Endocrinology and Metabolism, 65*, 494-498.

Zafrani, E. S. (2004). Non-alcoholic fatty liver disease: an emerging pathological spectrum. *Virchows Archiv, 444*, 3-12.

Zhang, Y., Hufnagel, C., Eiden, S., Guo, K.-Y., Diaz, P. A., Leibel, R. L., & Schmidt, I. (2001). Mechanism for LEPR-mediated regulation of leptin expression in brown and white adipocytes in rat pups. *Physiological Genomics, 4*, 189-199.

Zhang, Y., Olbort, M., Schwarzer, K., Nuesslein-Hildesheim, B., Nicolson, M., Murphy, E., Kowalski, T. J., Schmidt, I., & Leibel, R. L. (1997). The leptin receptor mediates apparent autocrine regulation of leptin gene expression. *Biochemical and Biophysical Research Communication, 240*, 492-495.

Zhou, G., Myers, R., Li, Y., Chen, Y., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J., & Moller, D. E. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *Journal of Clinical Investigation, 108*, 1167-1174.

Zirk, M. (2005). 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. [dissertation]. Winnipeg (Manitoba): University of Manitoba.

Zou, C. C., Liang, L., Hong, F., Fu, J. F., & Zhao, Z. Y. (2005). Serum adiponectin, resistin levels and non-alcoholic fatty liver disease in obese children. *Endocrine Journal, 52*, 519-524.

APPENDIX 1 – DATA TABLES FOR STUDY 1 (FIGURES 4-26)

Table A1-1 – Baseline and Endpoint Measurements in Lean and *fa/fa* Zucker Rats (Data for Figures 4-13)

	Lean Baseline	*fa/fa* Baseline	Lean Endpoint	*fa/fa* Endpoint	*Overall Genotype Mean*
Body Weight[3] (g)	130 ± 2	171 ± 3*	328 ± 5*	561 ± 13#†	
Absolute Liver Weight[3] (g)	5.0 ± 0.1	8.5 ± 0.3*	9.5 ± 0.3*	24.5 ± 1.0#†	
Adjusted Liver Weight[5,6]	10.8 ± 1.4	12.6 ± 1.1	7.1 ± 0.7	12.3 ± 3.0	*Lean: 8.9 ± 0.5* *fa/fa: 12.4 ± 1.0*
Serum Insulin[1] (pmol/L)	108 ± 12	1266 ± 136*	243 ± 30*	3991 ± 597#†	*Lean: 178 ± 22* *fa/fa: 2629 ± 432*
Serum C-peptide (pmol/L)[3]	292 ± 42	3388 ± 267	1069 ± 100	9902 ± 707	
Hepatic Lipid Concentration[2] (%)	4.73 ± 0.17	9.73 ± 0.92‡	5.17 ± 0.41	18.0 ± 2.5^†	
Serum FFA (mmol/L)[3]	0.744 ± 0.076	1.15 ± 0.06*	0.512 ± 0.047&	1.24 ± 0.05†	
Serum TAG[3,6] (mmol/L)	0.620 ± 0.050	5.55 ± 0.45	0.494 0.079	5.24 ± 1.09	*Lean:0.55 ± 0.05* *fa/fa: 5.39 ± 0.58*
Serum Adiponectin[4] (ng/mL)	3681 ± 395	6024 ± 213*	4080 ± 285	5134 ± 296•$	
Serum ALT[3] (U/L)	30.5 ± 1.5	67.8 ± 3.9*	30.0 ± 2.2	218 ± 29#†	

[1] Data are presented as means ± SEM (n=9 or 10 per group).
[2] Data are presented as means ± SEM (n=4, 6 or 10 per group).
[3] Data are presented as means ± SEM (n=10 per group).
[4] Data are presented as means ± SEM (n=8, 9 or 10 per group).
[5] Data are presented as adjusted means ± SEM, where liver weight was adjusted for body weight using ANCOVA (n=10 per group).
[6] Only a significant main effect of genotype was found ($p<0.0001$ for serum TAG and $p=0.01$ for adjusted liver weight). Therefore, overall genotype mean is also given.
* Significantly different from lean baseline ($p<0.001$).
# Significantly different from *fa/fa* baseline ($p<0.001$).

† Significantly different from lean endpoint ($p<0.001$).
‡ Significantly different from lean baseline ($p<0.001$).
& Significantly different from lean baseline ($p<0.05$).
^ Significantly different from *fa/fa* baseline ($p<0.05$).
• Significant trend towards different from *fa/fa* baseline ($p=0.0285$).
$ Significant trend towards different from lean endpoint ($p=0.0407$).

Table A1-2 – Total Feed Intake and Final Body Weights of Lean and *fa/fa* Zucker Rats fed CLA-containing or Control Diets for 8 Weeks (Data for Figures 14 and 15)

	9-11	10-12	TOG	BIO	NCK	CTL	*Overall Genotype Mean*[1]
Total Feed Intake[2] **(g)**							
Lean	959 ± 21	999 ± 17	1023 ± 23	976 ± 35	901 ± 17	1022 ± 24	
fa/fa	1562 ± 55	1399 ± 30#†	1539 ± 50	1516 ± 32*	1466 ± 35#	1665 ± 51	
Body Weight (g)							
Lean	336 ± 6	332 ± 9	327 ± 7	325 ± 8	317 ± 6	328 ± 6	*327 ± 3*
fa/fa	543 ± 13	545 ± 9	567 ± 18	534 ± 10	534 ± 14	561 ± 13	*547 ± 5*^

Data are presented as means ± SEM (n=10 per group).
[1] Overall genotype mean is presented if only a significant effect of genotype was found (body weight).
[2] There was a significant genotype x diet interaction ($p<0.0166$).
* Significantly different from *fa/fa* CTL ($p<0.05$).
# Significantly different from *fa/fa* CTL ($p<0.01$).
† Significantly different from *fa/fa* 9-11 ($p<0.05$).
^ Significant genotype difference ($p<0.0001$).

Table A1-3 – Absolute Liver Weight, Adjusted Liver Weight and Hepatic Lipid Concentration of Lean and *fa/fa* Zucker Rats fed CLA-containing or Control Diets for 8 Weeks (Data for Figures 16, 17 and 18)

	9-11	10-12	TOG	BIO	NCK	CTL
Absolute Liver Weight[1,4] (g)						
Lean	9.6 ± 0.3	9.8 ± 0.5	9.8 ± 0.32	9.7 ± 0.5	9.6 ± 0.3	9.5 ± 0.3
fa/fa	22.5 ± 1.1	17.5 ± 0.4[#‡]	17.3 ± 0.8[#‡]	17.6 ± 0.3[#‡]	17.5 ± 0.5[#‡]	24.5 ± 1.0
Adjusted liver Weight[2,4]						
Lean	13.0 ± 0.6	13.6 ± 0.7	13.6 ± 0.7	13.5 ± 0.7	13.8 ± 0.7	13.3 ± 0.7
fa/fa	18.8 ± 0.7	13.8 ± 0.7[#‡]	12.9 ± 0.7[#‡]	14.3 ± 0.6[#‡]	14.2 ± 0.6[#‡]	20.3 ± 0.7
Hepatic Lipid Concentration (%)[3,4]						
Lean	5.0 ± 0.4	5.5 ± 0.3	5.4 ± 0.4	4.8 ± 0.4	4.8 ± 0.6	5.2 ± 0.4
fa/fa	18.4 ± 2.4	6.3 ± 0.3[#‡]	6.6 ± 0.6[#‡]	9.0 ± 0.9[#†]	6.0 ± 0.4[#‡]	18.0 ± 2.5

[1] Data are presented as means ± SEM (n=10 per group).
[2] Data are presented as adjusted means ± SEM, where liver weights were adjusted for body weight using ANCOVA (n=10 per group).
[3] Data are presented as means ± SEM (n=4).
[4] There was a significant genotype x diet interaction ($p<0.001$ for absolute and adjusted liver weights; $p=0.004$ for hepatic lipid concentration).
[#] Significantly different from *fa/fa* CTL ($p<0.01$).
[†] Significantly different from *fa/fa* 9-11 ($p<0.05$).
[‡] Significantly different from *fa/fa* 9-11 ($p<0.01$).

Table A1-4 – Serum Total Cholesterol of Lean and *fa/fa* Zucker Rats fed CLA-containing or Control Diets for 8 Weeks (Data for Figure 19)

	9-11	10-12	TOG	BIO	NCK	CTL
Serum Total Cholesterol (mmol/L)						
Lean	11.2 ± 0.8	11.5 ± 0.9	11.6 ± 0.6	9.6 ± 0.58	11.1 ± 0.6	12.3 ± 0.6
fa/fa	28.2 ± 2.5	21.4 ± 1.4[#‡]	22.9 ± 1.8[#]	22.3 ± 1.5[#†]	23.3 ± 1.4[#]	30.5 ± 1.5

Data are presented as means ± SEM (n=10 per group).
There was a significant genotype x diet interaction (p=0.0114).
[#] Significantly different from *fa/fa* CTL (p<0.01).
[†] Significantly different from *fa/fa* 9-11 (p<0.05).
[‡] Significantly different from *fa/fa* 9-11 (p<0.01).

Table A1-5 – Five-hour Serum Fasting Glucose of Lean and *fa/fa* Zucker Rats fed CLA-containing or Control Diets for 8 Weeks (Data for Figure 20)

	9-11	10-12	TOG	BIO	NCK	CTL	*Overall Genotype Mean*
Serum Glucose (mmol/L)							
Lean	7.99 ± 0.43	7.65 ± 0.46	7.13 ± 0.28	7.83 ± 0.30	6.96 ± 0.36	7.60 ± 0.24	*7.52 ± 0.15*
fa/fa	9.09 ± 0.37	10.50 ± 0.7	8.98 ± 0.22	9.34 ± 0.35	9.10 ± 0.41	9.06 ± 0.76	*9.35 ± 0.21*^

Data are presented as means ± SEM (n=10 per group).
^ Significant genotype difference ($p<0.0001$).

Table A1-6 – Serum ALT and Haptoglobin in Lean and *fa/fa* Zucker Rats fed CLA-containing or Control Diets for 8 Weeks (Data for Figures 21 and 22)

	9-11	10-12	TOG	BIO	NCK	CTL
Serum ALT[1] (U/L)						
Lean	32 ± 1	34 ± 2.0	38 ± 3	43 ± 5	38 ± 2	30 ± 2
fa/fa	172 ± 23	111 ± 10$^{\#\ddagger}$	120 ± 5$^{\#\dagger}$	112 ± 10$^{\#\dagger}$	110 ± 8$^{\#\ddagger}$	218 ± 29
Serum Haptoglobin[2] (mg/mL)						
Lean	0.59 ± 0.08	0.85 ± 0.12	0.68 ± 0.12	0.69 ± 0.10	0.83 ± 0.16	0.77 ± 0.08
fa/fa	2.38 ± 0.18	1.42 ± 0.08*‡	1.55 ± 0.12‡	1.57 ± 0.09‡	1.43 ± 0.19*‡	2.04 ± 0.20

[1] Data are presented as means ± SEM (n=10 per group).
[2] Data are presented as means ± SEM (n=8 per group).
There was a significant genotype x diet interaction ($p<0.0001$ for both serum ALT and haptoglobin).
[#] Significantly different from *fa/fa* CTL ($p<0.05$).
[‡] Significantly different from *fa/fa* CTL ($p<0.01$).
[†] Significantly different from *fa/fa* 9-11 ($p<0.05$).

Table A1-7 – Hepatic Protein Levels of AMPK, pAMPKα, SREBP-1 p125 and SREBP-1 p68 (arbitrary units) in Lean and *fa/fa* Zucker Rats fed CLA-containing or Control Diets for 8 Weeks (Data for Figures 23-26)

	9-11	10-12	TOG	BIO	NCK	CTL	*Overall Genotype Mean*
AMPK							
Lean	3.13 ± 0.50	2.48 ± 0.48	1.89 ± 0.49	1.53 ± 0.51	2.11 ± 0.49	1.53 ± 0.48	*2.44 ± 0.20*
fa/fa	1.53 ± 0.48	1.49 ± 0.48	1.62 ± 0.48	1.71 ± 0.51	1.82 ± 0.48	1.72 ± 0.49	*1.65 ± 0.20*^
pAMPKα							
Lean	0.564 ± 0.167	0.392 ± 0.161	0.365 ± 0.161	0.417 ± 0.161	0.612 ± 0.161	0.601 ± 0.169	*0.492 ± 0.073*
fa/fa	0.339 ± 0.162	0.332 ± 0.163	0.353 ± 0.162	0.393 ± 0.161	0.352 ± 0.161	0.404 ± 0.161	*0.362 ± 0.073*
SREBP-1 p125							
Lean	1.77 ± 0.32	2.01 ± 0.30	1.77 ± 0.30	2.25 ± 0.31	2.34 ± 0.31	2.16 ± 0.30	*2.05 ± 0.12*
fa/fa	1.26 ± 0.30	1.73 ± 0.31	1.80 ± 0.30	1.56 ± 0.31	1.97 ± 0.30	1.80 ± 0.31	*1.69 ± 0.12*^Δ
SREBP-1 p68							
Lean	1.17 ± 0.23	1.29 ± 0.22	1.48 ± 0.22	0.81 ± 0.23	0.92 ± 0.22	1.39 ± 0.23	*1.14 ± 0.09*
fa/fa	1.39 ± 0.23	1.07 ± 0.23	1.05 ± 0.22	1.21 ± 0.23	1.17 ± 0.22	1.15 ± 0.22	*1.17 ± 0.09*

Data are presented as means ± SEM (n=4 per group).
^ Significant genotype difference (p=0.0127).
Δ Significant genotype difference (p=0.05).

APPENDIX 2 – DATA TABLES FOR STUDY 2 (FIGURES 27-36)

Table A2-1 – Total Feed Intake, Re-feed Intake and Final Body Weights of Lean and *fa/fa* Zucker Rats, in the Fed or Fasted State, Fed CLA-containing or Control Diets for 8 Weeks (Data for Figures 27, 28 and 29)

	fa 9-11 fed	fa 10-12 fed	fa CTL fed	ln CTL fed	fa CTL fasted
Total Feed Intake (g)	1615 ± 35	1619 ± 24	1524 ± 29	1190 ± 34*#	1641 ± 39
Re-feed Intake (g)	5.63 ± 0.70	5.20 ± 0.38	5.48 ± 0.40	5.40 ± 0.38	*N/A*[1]
Body Weight (g)	608 ± 9	598 ± 11	581 ± 10	357 ± 12*#	588 ± 14

Data are presented as means ± SEM (n=6 or 7 per group).
[1] *N/A* = not applicable.
* Significantly lower than fa CTL fed ($p<0.001$).
# Significantly lower than fa CTL fasted ($p<0.001$).

Table A2-2 – Absolute and Adjusted Liver Weights of Lean and *fa/fa* Zucker Rats, in the Fed or Fasted State, Fed CLA-containing or Control Diets for 8 Weeks (Data for Figures 30 and 31)

	fa 9-11 fed	fa 10-12 fed	fa CTL fed	ln CTL fed	fa CTL fasted
Absolute Liver Weight[1] (g)	23.5 ± 1.2	20.8 ± 0.6#	25.6 ± 0.9	11.9 ± 0.6*†	23.5 ± 0.9
Adjusted Liver weight[2]	20.1 ± 1.0#	18.0 ± 1.0*	23.6 ± 0.9	21.1 ± 0.9	21.8 ± 2.3

[1] Data are presented as means ± SEM (n=6 or 7 per group).
[2] Data are presented as adjusted means ± SEM (n=6 or 7 per group).
Statistics based on log transformed data.
Significantly lower than fa CTL fed ($p<0.01$).
* Significantly lower than fa CTL fed ($p<0.001$).

Table A2-3 – Hepatic Lipid Concentration of Lean and *fa/fa* Zucker Rats, in the Fed or Fasted State, Fed CLA-containing or Control Diets for 8 Weeks (Data for Figure 34)

	fa 9-11 fed	fa 10-12 fed	fa CTL fed	ln CTL fed	fa CTL fasted
Hepatic Lipid Concentration (%)	12.3 ± 0.7	6.4 ± 0.4$^{\#\dagger}$	13.9 ± 0.6	4.5 ± 0.2*	12.3 ± 2.8

Data are presented as means ± SEM (n=4 per group).
* Significantly lower than fa CTL fed ($p<0.01$).
# Significantly lower than fa CTL fed ($p<0.001$).
† Significantly lower than fa 9-11 fed ($p<0.05$).

Table A2-4 – Hepatic Protein Levels of AMPK, pAMPKα, SREBP-1 p125 and SREBP-1 p68 in Lean and *fa/fa* Zucker Rats, in the Fed or Fasted State, Fed CLA-containing or Control Diets for 8 Weeks (Data for Figures 33-36)

	fa 9-11 fed	fa 10-12 fed	fa CTL fed	ln CTL fed	fa CTL fasted
Hepatic AMPK[1] (arbitrary units)	1.68 ± 0.30	0.57 ± 0.30	0.81 ± 0.30	1.12 ± 0.30	0.66 ± 0.30
Hepatic pAMPKα (arbitrary units)	0.83 ± 0.22	1.07 ± 0.22	1.21 ± 0.20	0.96 ± 0.20	1.10 ± 0.21
Hepatic SREBP-1 p125[2] (arbitrary units)	2.52 ± 0.73*	2.69 ± 0.73#	3.11 ± 0.72	0.92 ± 0.73	2.91 ± 0.73
Hepatic SREBP-1 p68 (arbitrary units)	1.83 ± 0.47	1.24 ± 0.46	1.06 ± 0.46	2.79 ± 0.46	1.19 ± 0.46

Data are presented as adjusted means ± SEM (n=6 per group).
[1] Trend towards a difference in group (p=0.081).
[2] Significant effect of group (p=0.0179).
* Trend towards being significantly higher than fa CTL fed (p=0.015).
# Trend towards being significantly higher than fa CTL fed (p=0.0573).

APPENDIX 3

CHARACTERIZING FEED INTAKE PATTERNS IN LEAN AND *fa/fa* ZUCKER RATS OVER A 24-HOUR PERIOD

Introduction

Rats are nocturnal animals. Under natural conditions, rats consume feed during the night. However, different conditions in animal holding facilities may affect normal behavioural patterns of rats. The objective of this study was to characterize the 24-hour feeding pattern of lean and *fa/fa* Zucker rats housed in the Animal Holding Facility at the University of Manitoba, which employs a 14-hour light, 10-hour dark cycle. In this facility, lights are turned off at 9 p.m. and are turned back on at 7 a.m.

Methods

Ten lean and 10 *fa/fa* Zucker rats were studied for 48 hours. Rats were initially fed at 9 a.m. The initial weights of the feed dishes (feed included) were recorded. Subsequently, dish weights were recorded at 3 hour intervals for 48 hours. This allowed two measurements for each 3 hour interval (i.e. 2 measurements for 9 a.m. to 12 p.m.; two measurements for 12 p.m. to 3 p.m., etc.). Intakes of lean and *fa/fa* Zucker rats during the 3-hour intervals were calculated as the average feed intake of the two measurements. To estimate the rate of feed intake of lean and *fa/fa* Zucker rats, linear regression was used to find the best fit line between time of day and weight of feed dishes for both lean and *fa/fa* Zucker rats. The slope of the best fit line (represented by "m") was used as the estimate of the rate of feed intake. Linear regression was performed

using Origin software version 6.0 (Microcal Software, Inc., Northhampton, MA). Student's *t*-test, calculated by hand, was used to determine if the slopes were significantly different. Student's t-test was also used to test for differences in total feed intake over 48 hours, using Origin software version 6.0 (Microcal Software, Inc., Northhampton, MA).

Results

The rate of feed intake was significantly higher (p=0.001) in *fa/fa* Zucker rats than in lean Zucker rats (Figure A3-1). As anticipated, total feed intake was higher in *fa/fa* than in lean rats (Figure A3-2).

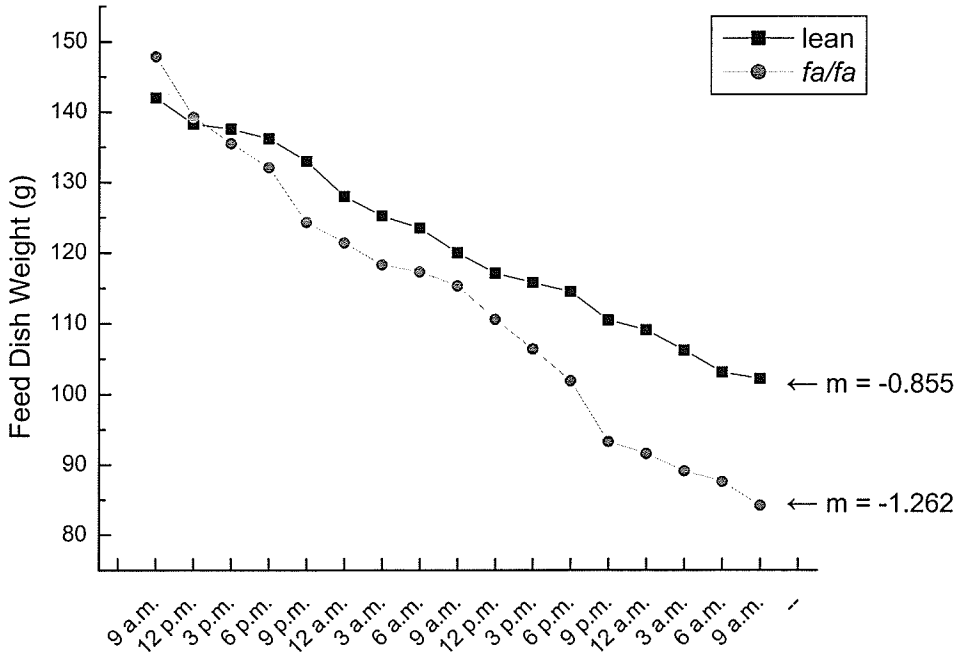

Figure A3-1. Feed dish weights during 48 hours of monitoring feed consumption. Data are expressed as means for each time point (n=10 per group).

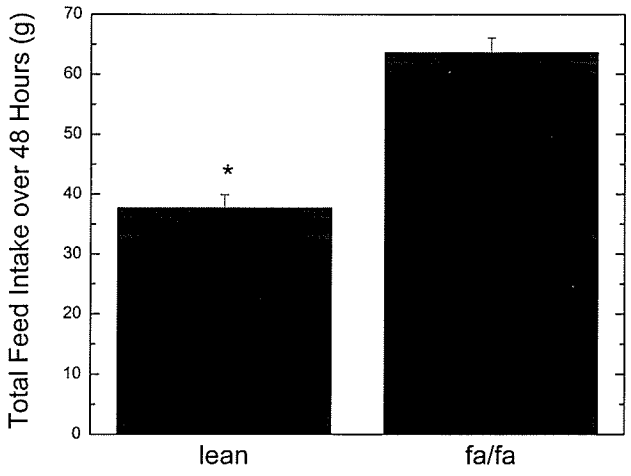

Figure A3-2. Total feed intake over 48 hours in lean and fa/fa Zucker rats. The * denotes a significant difference ($p<0.05$). Data are presented as means ± SEM (n=10 per group).

Feed intake in both lean and *fa/fa* Zucker rats was highest between 6 p.m. and 9 p.m. (Figure A3-3). In lean rats, the period of lowest feed intake was between 12 p.m. and 3 p.m., whereas for *fa/fa* rats, the period of lowest feed intake was between 3 a.m. and 6 a.m.

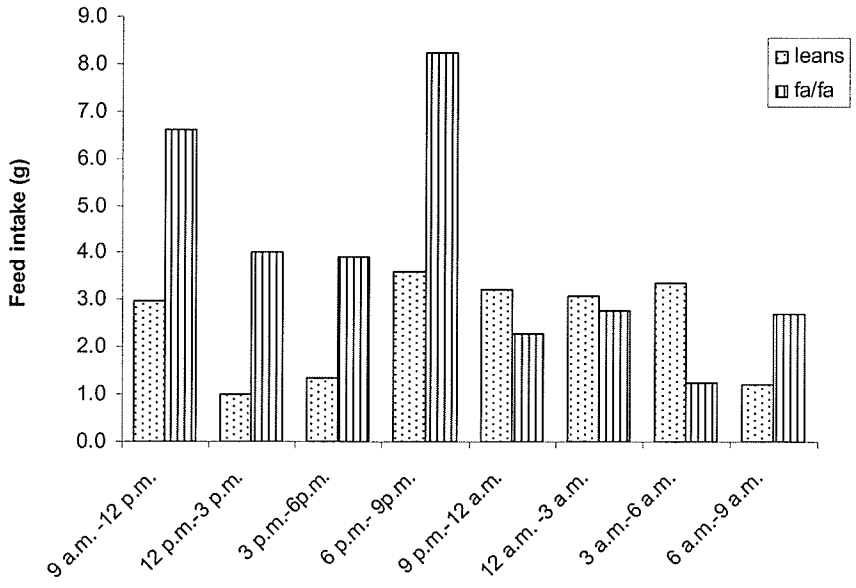

Figure A3-3. Twenty-four hour feed intake in lean and *fa/fa* Zucker rats. Data are presented as means (n=10 per group).

Discussion

Rats, which are naturally nocturnal, eat approximately 75% of their total daily feed intake at night (Rosenwasser et al., 1981). However, interruption of the normal day-night cycle imposed by artificial light-dark cycles used in animal holding facilities could alter their normal feeding patterns.

Feed intake was monitored at 3-hour intervals for 48 hours. Thus, we separated feed intake into 3-hour blocks of time (i.e. 9 a.m. to 12 p.m., 12 p.m. to 3 p.m., etc). Each 3-hour block of time was measured twice to calculate the average feed intake for the block of time (i.e. feed intake between 9 a.m. and 12 p.m. was measured on two days and averaged to yield an average feed intake between that time period). As expected, *fa/fa* rats consumed more food over a 24-hour period than lean rats (31.8 g vs. 19.8 g, respectively). During the light period, feed intake of the *fa/fa* rats was 60% higher than that of lean rats (25.5 g vs. 10.2 g, respectively); however, during the dark period, feed intake of the lean rats was 34.7% higher that that of the *fa/fa* rats (9.65 g vs. 6.30 g, respectively).

Feed intake during the light period of the lean rats represented 48% of their total feed intake over 24-hours. In contrast, the feed intake of the *fa/fa* rats during the light period represented only 19.8% of their total feed intake over 24-hours. Feed intake during the dark period of lean rats represented 51.3% of their total feed intake over 24-hours, while feed intake of *fa/fa* rats during this period represented 80.3% of total feed intake over 24-hours. Based on these

observations, it appears that feed intake of lean Zucker rats is more affected by artificial housing conditions than is feed intake of *fa/fa* Zucker rats.

In our laboratory, termination procedures begin at 9 a.m. Therefore, time of feeding could have implications for studying parameters in the fed state. We were curious to see if rats ate a substantial amount of feed in the 3-hour period immediately preceding termination time of our laboratory. If rats do not have substantial feed intake during this time, measurements made in tissues of these animals might not truly reflect the fed state levels. Based on our observations, it appears that both lean and *fa/fa* Zucker rats eat a relatively low amount of feed in this time period. Additionally, it is unknown if the rats consumed feed closer to the termination time of 9 a.m. or closer to 6 a.m. Therefore, a marker of fed state activation in the liver should be measured in rats allowed feed *ad libitum* overnight to assess their nutritional state.

References

Rosenwasser, A. M., Boulos, Z., & Terman, M. (1981). Circadian organization of food intake and meal patterns in the rat. *Physiology and Behavior, 27*, 33-39.

APPENDIX 4

EXPRESSION AND ACTIVATION OF

SELECTED HEPATIC PROTEINS IN THE FED VS. FASTED STATE

Introduction

The nutritional state (i.e. fasted state or fed state) influences the expression and activation of proteins that are key mediators for glucose, protein, and lipid metabolism. For example, activation of intermediates in the insulin signaling cascade is up-regulated in the fed state, but down-regulated in the fasted state. Insulin binding to its receptor causes phosphorylation of IRS-1 on tyrosine residues (pIRS-1^{tyr}), which in turn causes phosphorylation and activation of Akt (Lizcano & Alessi, 2002). One of the many functions of Akt is the regulation of glycogen synthesis through phosphorylation and inactivation of glycogen synthase kinase-3α/β (GSK3α/β) (Hadjuch et al., 2001; Cross et al., 1995). Another arm of the pIRS-1 pathway involves phosphorylation of mitogen-activated protein kinase (MAPK), a protein kinase involved in cell growth. Therefore, the objective of this study was to measure and compare hepatic levels of pIRS-1^{tyr}, phosphorylation of Akt on serine residues (pAkt^{ser}), phosphorylation of Akt on threonine resides (pAkt^{thr}), phosphorylation of MAPK (pMAPK), and phosphorylation of GSK3α/β (pGSK3α/β) in the fasted and fed states. As results from this study were to be used for designing a study examining the effects of dietary CLA on activation of AMPK and SREBP-1 in the fed state, levels of AMPK, pAMPKα, SREBP-1 p125, and SREBP-1 p68 were also measured.

Methods and Statistical Analysis

Four lean Zucker rats were allowed *ad libitum* access to feed overnight, while 4 lean Zucker rats were fasted overnight. Starting at 9 a.m. the next morning, rats were sacrificed by carbon dioxide asphyxiation and cervical dislocation. Livers were removed, rinsed in cold PBS, wrapped in foil and immediately snap-frozen in liquid nitrogen until storage at -80°C. Hepatic protein extraction, quantification, SDS-PAGE and Western immunoblotting were carried out as in the Methods section of this dissertation (see page 74). Hepatic levels of AMPK, pAMPKα, pIRS-1^{tyr}, pMAPK, pAkt^{ser}, pAkt^{thr}, pGSK3α/β, SREBP-1 p125, and SREBP-1 p68 were measured. Antibodies used for western immunoblotting are listed in Table A3-1.

For all proteins measured, values were expressed relative to levels of AMPK to control for loading variation. In addition, pAMPKα and SREBP-1 p68 values were expressed relative to total hepatic AMPK and SREBP-1, respectively. T-tests were performed using Origin software version 6.0 (Microcal Software, Inc., Northhampton, MA). Differences having a p-value of 0.05 or less were considered significant.

Table A4-1 – Antibodies Used for Western Immunoblotting (Lean Zucker Rat Fed vs. Fasted Study)

Primary Antibody	Name	Source	Dilution[1]	Host	Secondary Antibody	Dilution[2]	Molecular Weight (kDa)
pAkt serine	Phospho-Akt (ser437)	Cell Signaling, Cat. # 9271	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	60
pAkt threonine	Phospho-Akt (thr308)	Cell Signaling, Cat. # 9275	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	60
AMPK	AMP-activated protein kinase	Cell Signaling, Cat. # 2532	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	62
pAMPKα	Phospho-AMPK-α (thr172)	Cell Signaling, Cat. # 2531	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	62
pGSK3α/β	Phospho-GSK-3α/β (ser21/9)	Cell Signaling, Cat. # 9331	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	46 (α) 52 (β)
pIRS-1	Phospho-IRS-1 (tyr941)	Oncogene Research Products, Cat. # GF1004	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	165
pMAPK	Phospho-MAPK p44/p42 (thr202/Tyr204)	Cell Signaling, Cat. # 9101	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	42, 44
SREBP-1	Sterol regulatory element-binding protein 1	Santa Cruz Biotechnology, Inc.,	1:500	Rabbit	Anti-rabbit horseradish peroxidase[4]	1:10 000	125 (inactive)

	(C-20)	Cat. # sc-366					68 (active)

[1] Primary antibodies diluted with 3% BSA-TBST.
[2] Secondary antibodies diluted with 1% BSA-TBST.
[3] Membranes incubated in secondary antibody for 1 hour.
[4] Membranes incubated in secondary antibody for 45 minutes.

Results

There were no significant differences in AMPK, pAMPKα, pMAPK, $pAkt^{ser}$, $pAkt^{thr}$, or pGSK3α/β between the fasted and fed lean Zucker rats (Figures A4-1 and A4-2). There was a trend (p=0.06554) for levels of $pIRS\text{-}1^{tyr}$ to be higher in fed rats than in fasted rats (Figure A4-1). Full-length SREBP-1 p125 was significantly higher and SREBP-1 p68 was significantly lower in the fed lean Zucker rats compared to the fasted lean Zucker rats (Figure A4-3).

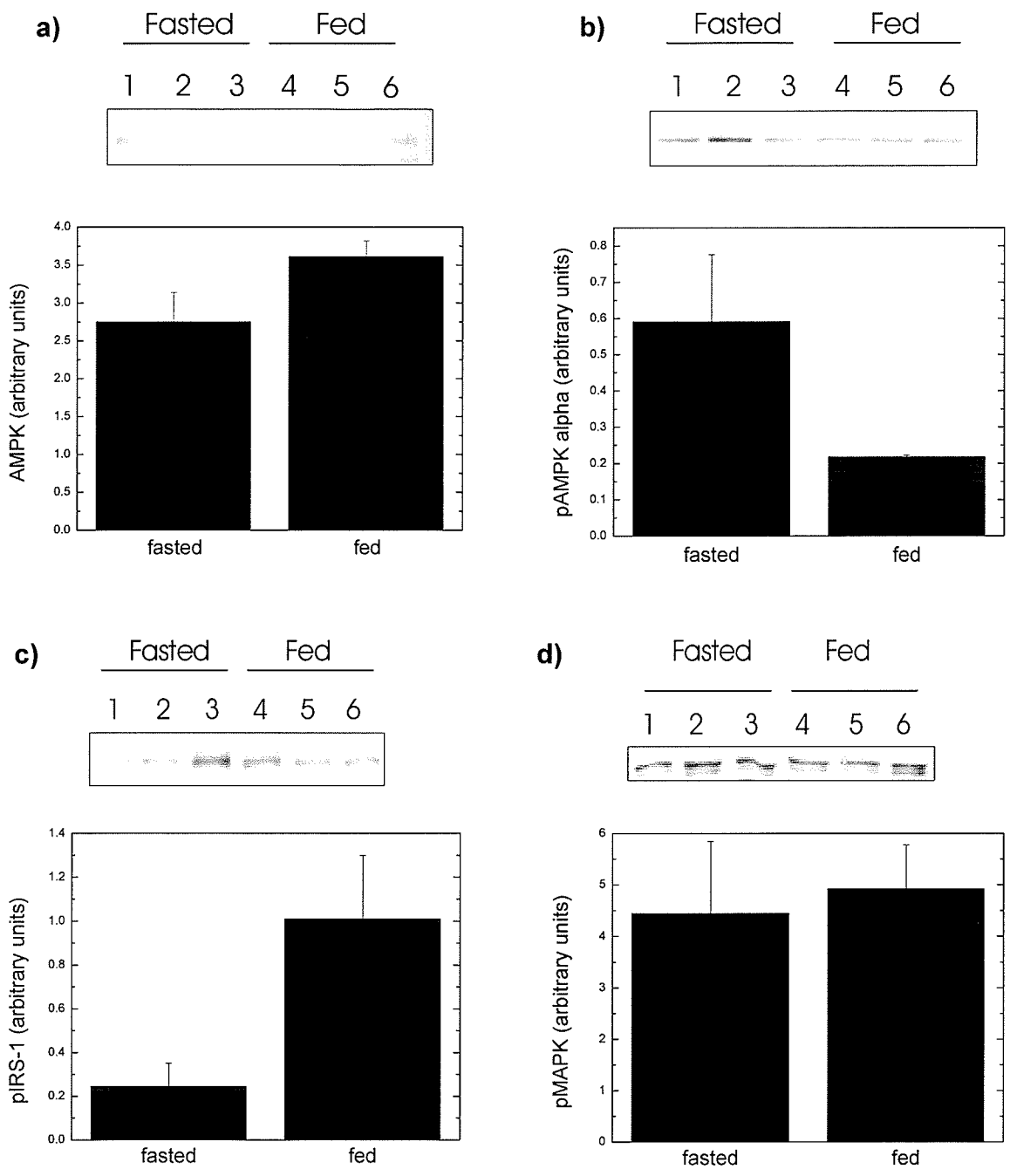

Figure A4-1. Hepatic levels of a) AMPK, b) pAMPKα, c) pIRS-1^{tyr} and d) pMAPK in fasted and fed lean Zucker rats. There were no significant differences in AMPK, pAMPKα, and pMAPK between fasting and feeding. There was a trend (p=0.06554) for pIRS-1 to be higher in fed rats. Data are presented as mean ± SEM (n=3 per group).

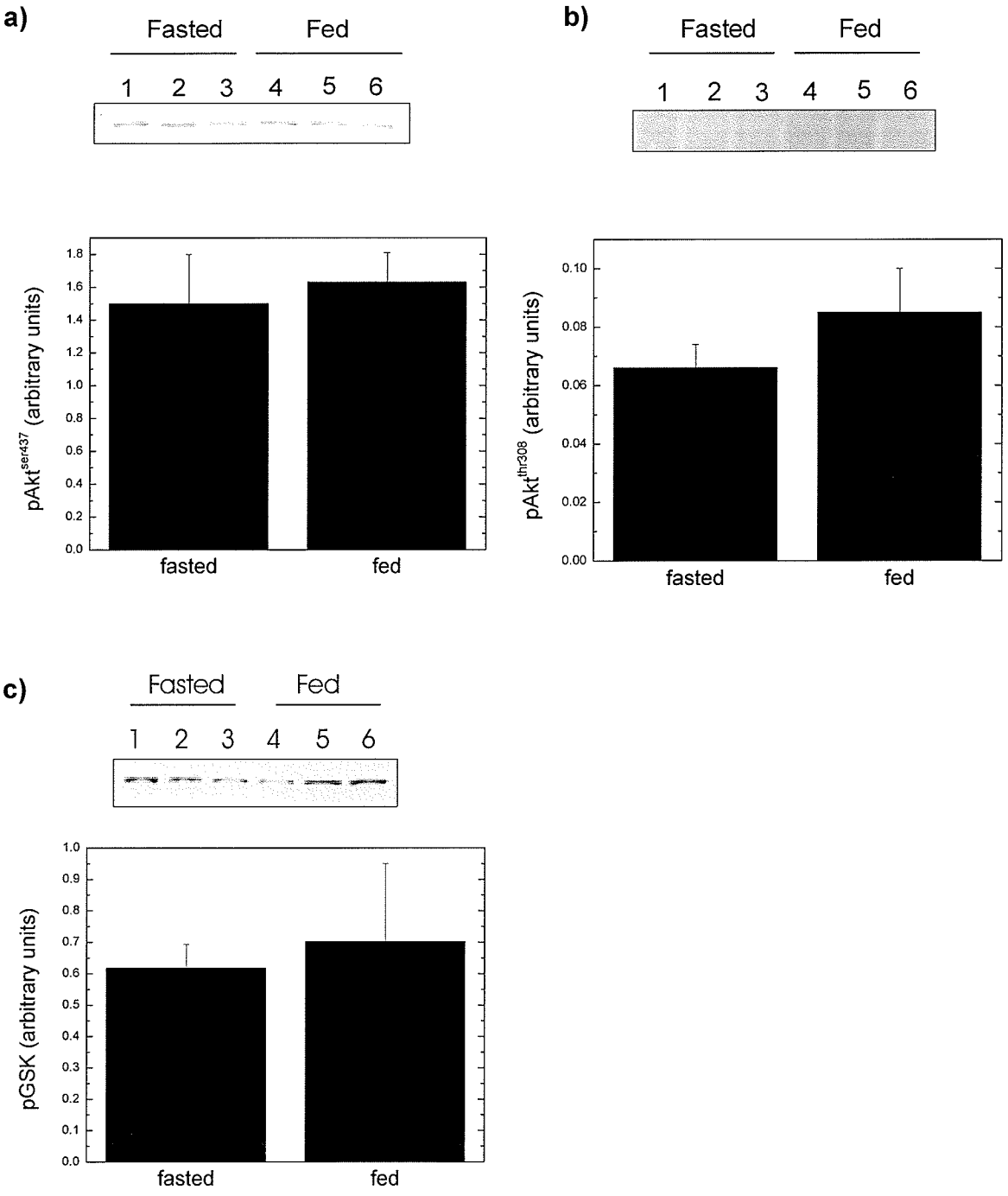

Figure A4-2. Hepatic levels of a) pAkt^{ser437}, b) pAkt^{thr308} and c) pGSK3α/β in fasted and fed lean Zucker rats. There were no differences between fasted and feeding. Data are presented as mean ± SEM (n=3 per group).

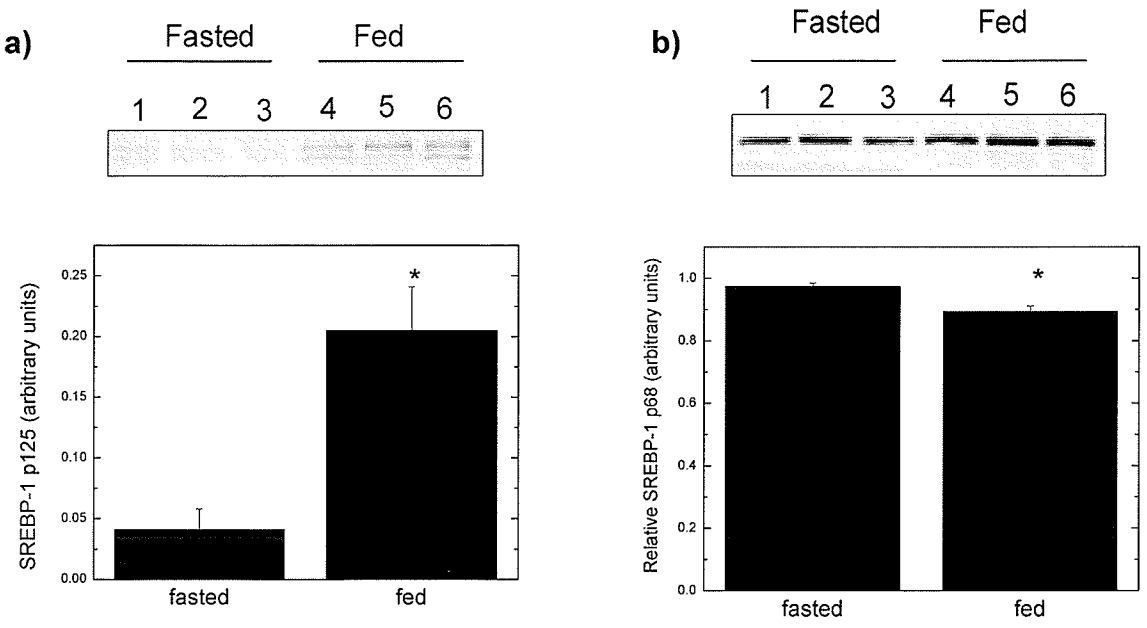

Figure A4-3. Hepatic levels of a) SREBP-1 p125 and b) SREBP-1 p68 (p68/p68+p125) in fasted and fed lean Zucker rats. The * denotes a significant difference from fasted ($p<0.05$). Data are presented as mean ± SEM (n=3 per group).

Discussion

In the fed state, elevations in blood insulin concentrations initiate a cascade of phosphorylation reactions that mediate glucose, lipid, and protein metabolism. We expected to see higher hepatic levels of pIRS-1^{tyr}, pAkt^{ser}, pAkt^{thr}, pAMPKα, pMAPK, and pGSK3α/β in the rats allowed access to feed overnight. However, we observed no significant differences between the fasted and fed groups in any of these proteins. Although not significant, there was a trend ($p=0.06554$) for pIRS-1^{tyr}, a marker of positive insulin signaling, to be higher in fed rats compared to fasted rats (Figure A3-3c). Perhaps a larger sample size would have yielded significant results.

Surprisingly, pAMPKα was not different between fasting and feeding. AMPKα phosphorylation and activation are sensitive to the nutritional status of the animal, as AMPK activation conserves energy stores in fasting animals by inhibiting ATP-consuming reactions (Carling, 2004). Once again, however, pAMPKα levels appeared to be higher in fasted rats based on means, suggesting that a higher sample size might have yielded significant results.

SREBP-1 p125 levels were significantly higher in livers of fed rats compared to fasted rats (Figure A3-5a). This finding was expected as insulin has been shown to regulate full-length SREBP-1 expression (Cagen et al., 2005). Unexpectedly, levels of active SREBP-1 p68 were lower in fed animals than in fasted animals. Insulin also positively regulates activation of SREBP-1 in rat hepatocytes (Yellatune et al., 2005); therefore we expected higher levels of

SREBP-1 p68 in the fed state, where blood insulin levels are higher than the fasted state.

However unexpected, these results provided an insight into how nutritional status affects levels of hepatic proteins involved in glucose, lipid and protein metabolism in lean Zucker rats. A similar experiment in *fa/fa* Zucker rats a model for hepatic insulin resistance, would also prove useful.

References

Cagen, L. M., Deng, X., Wilcox, H. G., Park, E. A., Raghow, R., & Elam, M. B. (2005). Insulin activates the rat sterol regulatory element-binding protein-1c (SREBP-1c) promoter through the combinatorial actions of SREBP, LXR, Sp1, and NF-Y cis-acting elements. *Biochemical Journal, 385*, 207-216.

Cross, A. E., Alessi, D. R., Cohen, P., Andjelkovich M., & Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature, 378*, 785-789.

Hajduch, E., Litherland, G. J., & Hundal, H. S. (2001). Protein kinase B (PKB/Akt) – a key regulator of glucose transport? *FEBS Letters, 493*, 199-203.

Yellatune, C. R., Deng, X., Cagen, L. M., Wilcox, H. G., Park, E. A., Raglow, R., & Elam, M. B. (2005). Posttranslational processing of SREBP-1 in rat hepatocytes is regulated by insulin and cAMP. *Biochemical and Biophysical Research Communications, 332*, 174-180.

Novell-Fernandez, J. M., Castel, S., Bellido, D., Ferrer, J. C., Vilaro, S., & Guinovart, J. J. (1999). Intracellular distribution of hepatic glucokinase and glucokinase regulatory protein during the fasted to re-fed transition in rats. *FEBS Letters, 459*, 211-214.

Yellatune, C. R., Deng, X., Cagen, L. M., Wilcox, H. G., Park, E. A., Raglow, R., & Elam, M. B. (2005). Posttranslational processing of SREBP-1 in rat hepatocytes is regulated by insulin and cAMP. *Biochemical and Biophysical Research Communications, 332*, 174-180.

APPENDIX 5

COMPARISON OF HEPATIC IRS-1 PHOSPHORYLATION IN LIVERS OF *AD LIBITUM*-FED OR RE-FED RATS

This set of experiments was designed to determine the most appropriate feeding regimen in which to examine the effects of dietary CLA on fed-state levels of SREBP-1 and AMPK activation. The main concern that prompted the initiation of these experiments was the possibility that rats allowed *ad libitum* access to feed the night preceding terminations might not eat at a time period preceding termination to evaluate their tissues in the fed state. As such, we compared the pIRS-1^{tyr} signal in livers of rats that had been allowed *ad libitum* access to feed overnight to rats that had been fasted for 12 hours and re-fed for 2 hours.

Methods

To compare hepatic pIRS-1^{tyr} levels in the fasted vs. fed state, four male *fa/fa* Zucker rats were allowed ad libitum access to feed overnight, while 4 male *fa/fa* Zucker rats were fasted overnight. Starting at 9 a.m. the next morning, rats were euthanized by carbon dioxide asphyxiation and cervical dislocation. Livers were removed, rinsed in cold PBS, wrapped in foil, and immediately snap frozen in liquid nitrogen until storage at -80°C. Hepatic protein extraction, quantification, SDS-PAGE, gel transfer, and Western Immunoblotting were carried out as in the Methods section of this dissertation (see page 74). The antibody against pIRS-1^{tyr} was the same as that used in Appendix 4.

In a separate experiment, 3 male Sprague Dawley rats were fasted for 12 hours and then re-fed for 2 hours. A 2-hour re-feeding period was chosen based on published studies that have shown maximal activity of glucokinase, a glycolytic enzyme and hepatic glucose sensor, 2 hours after restoration of feed after an overnight fast (Fernandez-Novell et al., 1999). After the 2-hour feeding period was over, rats were euthanized via carbon dioxide asphyxiation and cervical dislocation. Livers were removed, rinsed in cold PBS, wrapped in foil, and immediately snap frozen in liquid nitrogen until storage at -80°C. Hepatic protein extraction, quantification, SDS-PAGE, gel transfer and western immunoblotting were carried out as in the Methods section of this dissertation (see page 74). The antibody against pIRS-1^{tyr} was the same as that used in Appendix 4. Three fasted lean Zucker rat liver samples were run on the same gel for reference.

Results and Discussion

Although levels of hepatic pIRS-1^{tyr} were higher in fed *fa/fa* rats compared to fasted *fa/fa* rats, this difference was not statistically significant (Figure A5-1). Fasted *fa/fa* Zucker rats had almost undetectable levels of hepatic pIRS-1^{tyr}, except for rat #3. Despite being in the fed state, 2 of the 4 fed *fa/fa* Zucker rats did not display a strong pIRS-1^{tyr} signal.

There was a trend for hepatic pIRS-1^{tyr} levels to be higher in re-fed Sprague Dawley rats than in fasted lean Zucker rats (p=0.083) (Figure A5-2). Liver extracts from each Sprague Dawley rat exhibited a strong pIRS-1^{tyr} signal.

This set of experiments was designed to determine the most appropriate feeding regime in which to examine the effects of dietary CLA on fed-state levels of SREBP-1 and AMPK activation. The main concern prompting the initiation of these experiments was that rats allowed *ad libitum* access to feed the night preceding terminations would not have eaten in a period of time before termination to be biochemically in the fed state. As such, we decided to compare the pIRS^{tyr} signal in livers of rats that had been allowed *ad libitum* access to feed overnight with rats that had been fasted for 12 hours and re-fed for 2 hours. In the first experiment, levels of pIRS-1^{tyr} were higher in fed rats than fasted rats; however, this difference was not significant. Furthermore, 2 of the 4 fed rats did not display a signal for pIRS-1, suggesting that these rats were not in the fed state at the time of termination, essentially cutting the fed-state sample size in half. When rats were re-fed after a 12-hour fast, there was a trend for pIRS-1^{tyr} levels to be higher than fasted rats. More importantly, all 3 of the re-fed rats displayed a strong pIRS-1 signal.

Therefore, to ensure that all rats are in the biochemical fed state, it was decided to employ a 12-hour fast followed by a 2-hour re-feed for the second dietary CLA study examining fed state activation of SREBP-1 and AMPK.

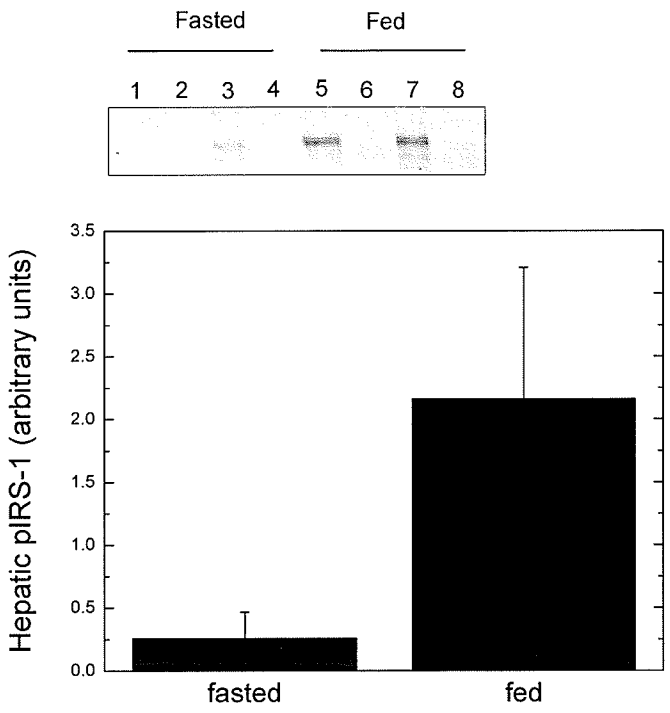

Figure A5-1. Hepatic pIRS-1^{tyr} levels in fasted and fed *fa/fa* Zucker rats. There was no significant difference between the two groups. Data are presented as mean ± SEM (n=4 for per group).

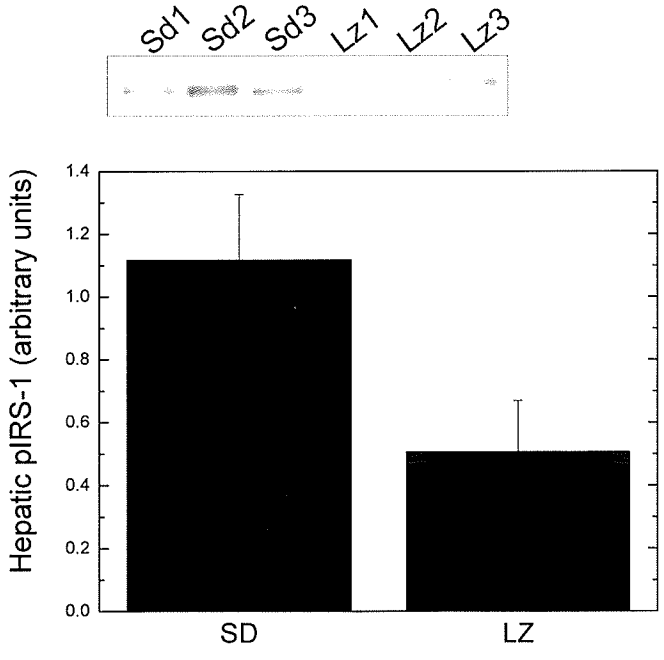

Figure A5-2. Hepatic pIRS-1^{tyr} levels in re-fed Sprague Dawley (Sd) rats. There was a trend for higher pIRS-1 in re-fed SD rats (p=0.082). Lz=Lean Zucker rat. Data are presented as mean ± SEM (n=3 per group).

References

Novell-Fernandez, J. M., Castel, S., Bellido, D., Ferrer, J. C., Vilaro, S., & Guinovart, J. J. (1999). Intracellular distribution of hepatic glucokinase and glucokinase regulatory protein during the fasted to re-fed transition in rats. *FEBS Letters, 459*, 211-214.

APPENDIX 6

EFFECTS OF DIETARY CLA ISOMERS ON LEVELS OF SELECTED PROTEINS INVOLVED IN HEPATIC GLUCOSE METABOLISM IN *fa/fa* ZUCKER RATS

Introduction

Hepatic glucose uptake accounts for 1/3 of post-prandial glucose disposal (Meyer et al., 2002). Within the hepatocyte, glucose is phosphorylated to form glucose 6-phosphate by glucokinase (GK), which has a high capacity for glucose. The properties of GK coupled with GLUT2 (the predominant glucose transporter in the liver) result in rapid uptake of glucose into the hepatocyte for phosphorylation when portal concentrations of glucose rise. Therefore, the ability of the liver to take up glucose is largely determined by the amount and activity of GK.

Short-term regulation of GK activity occurs via the GK regulatory protein (GKRP), a 68 kDa protein located in the nucleus of hepatocytes (Gregori et al., 2006; Van Schaftingen et al., 1994). Under conditions of low glucose, GK is sequestered in the nucleus of the hepatocyte by GKRP. Elevated glucose in the hepatocyte initiates a conformational change in GKRP, causing GK to be released from GKRP and translocated to the cytosol for phosphorylation of glucose. Any alteration in GK translocation results in impaired hepatic glucose uptake.

Carbohydrate response element-binding protein (ChREBP) is a 95 kDa transcription factor for enzymes involved in both glucose and lipid metabolism (Yamashita et al., 2001; Ishii et al., 2004). Target genes of ChREBP include liver pyruvate kinase, ACC, and FAS (Dentin et al., 2005; Stoeckman et al., 2004). Under conditions of low glucose, ChREBP is localized in the cytosol of the

hepatocyte and is inactive. Under conditions of high glucose, ChREBP is translocated into the nucleus, thereby allowing its binding to carbohydrate response elements present on glycolytic and lipogenic gene promoters. This translocation and activation of ChREBP involves two dephosphorylation steps: dephosphorylation of serine^{196} in the cytosol, which allows nuclear translocation, and dephosphorylation of threonine^{666} in the nucleus to allow DNA binding (Dentin et al., 2005). These two key dephosphorylation reactions are catalyzed by protein phosphatase 2A, which is selectively activated by xylulose 5-phosphate (an intermediate of the non-oxidative branch of the pentose phosphate pathway). GK has an indirect role in activation of ChREBP by phosphorylation of glucose for entry into the pentose phosphate pathway with subsequent generation of xylulose 5-phosphate (Weickert & Pfeiffer, 2006).

It has been established that CLA improves oral glucose tolerance in the *fa/fa* Zucker rat; specifically, it is the t10,c12 isomer that is responsible for this effect (Houseknecht et al., 1998; Ryder et al., 2001; Diakiw, 2005). In pre-diabetic ZDF rats, GK translocation is impaired (Fujimoto et al., 2004). *Fa/fa* Zucker rats, also in a "pre-diabetic" state, may also have impaired GK translocation. Thus, restoration of GK activity may be the mechanism behind improved oral glucose tolerance observed with dietary t10,c12 CLA. Furthermore, higher GK activity produces more xylulose 5-phosphate, activating protein phosphatase 2A and causing more activation of ChREBP.

The effects of CLA isomers on GK, GKRP, or ChREBP activity have not been determined previously. Furthermore, the effects of CLA isomers on levels

of these proteins have not been determined. Therefore, the aim of this preliminary experiment was to assess the effects of dietary CLA isomers on hepatic protein levels of GK, GKRP and ChREBP.

Methods

In an one-time experiment, hepatic protein extracts from one fa 9-11, fa 10-12, fa TOG, fa BIO, fa NCK, and fa CTL in Study 1 of this dissertation were taken and subjected to SDS-PAGE, gel transfer and Western immunoblotting (see Methods section of Study 1 for procedures). Antibodies used in Western immunoblotting are presented in Table A6-1.

Table A6-1 – Antibodies Used for Western Immunoblotting (Hepatic Glucose Metabolism Study)

Primary Antibody	Name	Source	Dilution[1]	Host	Secondary Antibody	Dilution[2]	Molecular Weight (kDa)
GK	GCK (C-20)	Santa Cruz Biotechnology, Inc., Cat. # sc-1979	1:1000	Goat	Anti-goat horseradish peroxidase[3]	1:10 000	50
GKRP	GCKR (N-19)	Santa Cruz Biotechnology Inc., Cat # sc-6340	1:1000	Goat	Anti-goat horseradish peroxidase[3]	1:10 000	68
ChREBP	ChREBP (P-13)	Santa Cruz Biotechnology, Inc., Cat. # sc-21189	1:1000	Goat	Anti-goat horseradish peroxidase[4]	1:10 000	95

[1] Primary antibodies diluted with 3% BSA-TBST
[2] Secondary antibodies diluted with 1% BSA-TBST
[3] Membranes incubated in secondary antibody for 1 hour

Results

GK levels were highest in the fa NCK rat. Levels of GK were intermediate in the fa 10-12, fa TOG, and fa BIO rats, while the lowest GK level was found in the fa 9-11 rat (Figure A4-1).

Hepatic protein expression of GKRP was highest in the fa 10-12 rat (Figure A4-2). Levels of GKRP were not as high in rats fed the other t10,c12-containing diets. GKRP protein expression was lowest in the fa 9-11 rat.

Hepatic levels of ChREBP were highest in the fa BIO rat and lowest in the fa CTL rat (Figure A4-3). Levels of ChREBP in livers of the fa 9-11 and fa 10-12 rat were almost equal.

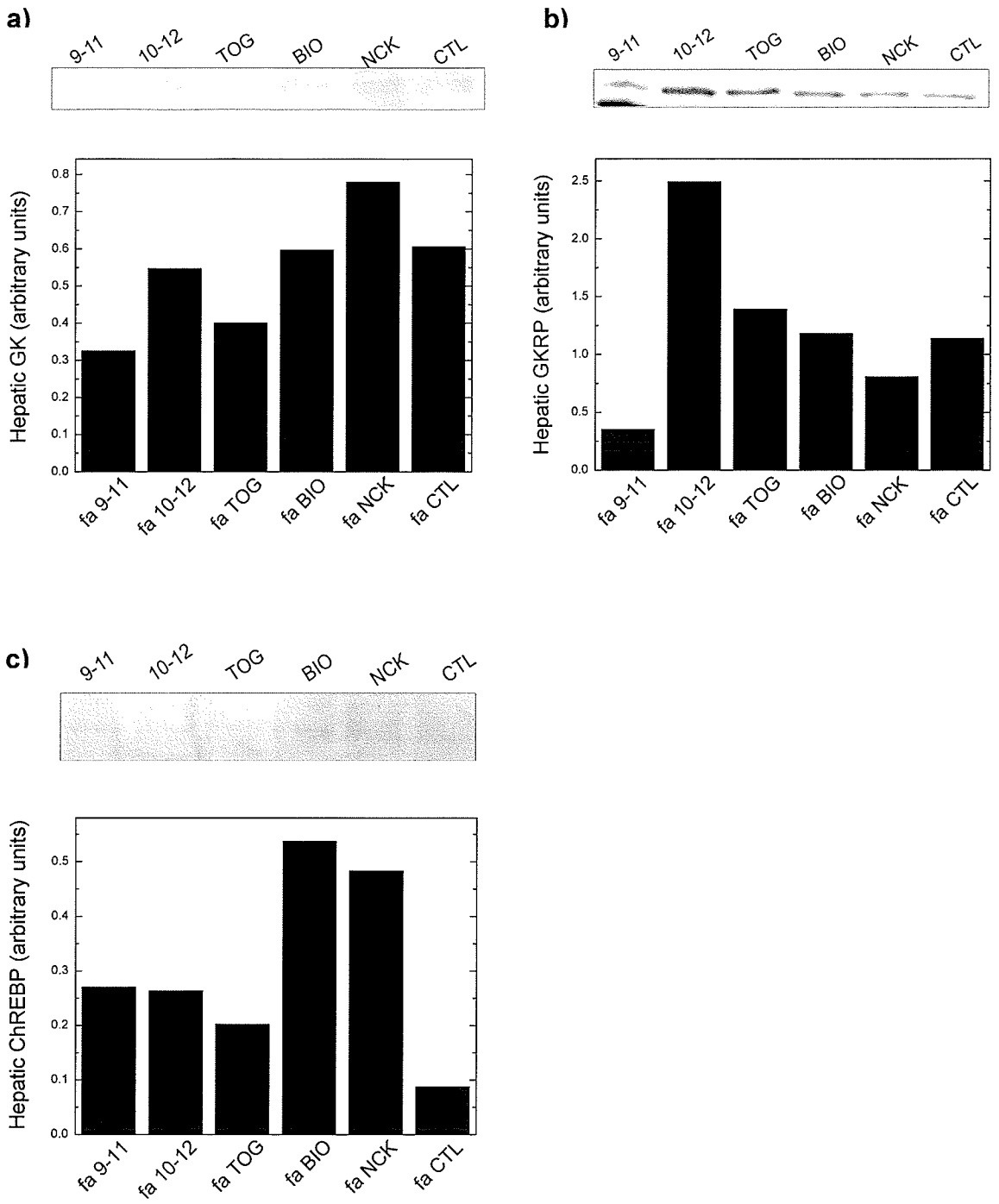

Figure A6-1. Hepatic levels of a) GK, b) GKRP and c) ChREBP in *fa/fa* Zucker rats fed CLA isomers for 8 weeks. Data are presented as single observations (n=1 per group).

Discussion

The pattern of GK protein levels in *fa/fa* rats fed t10,c12 CLA was not consistent with results from oral glucose tolerance testing performed on the same rats, which showed that all diets containing t10,c12 CLA improved glucose tolerance to the same degree (Diakiw, 2005). If increased levels of GK were responsible for improved glucose disposal, GK levels in fa 9-11 and fa CTL rats should be the same, while levels in the fa 10-12, fa TOG, fa BIO and fa NCK rats should be higher. It appears that t10,c12 CLA elevated levels of GKRP; however, rats fed the TOG, BIO and NCK diets did not have the same levels of GKRP as seen in the fa 10-12 rat. Likewise, there appeared to be no consistent pattern of hepatic ChREBP protein levels in rats fed diets containing the t10,c12 isomer.

There are many important factors to consider when interpreting the results of this one-time study. First, only one animal per dietary group was used; therefore, it is impossible to say the patterns observed in this study would be the same as if we repeated the study with a higher number of animals per group. The fact that we only used 1 animal per group makes it impossible to compute a standard deviation and standard error, and we cannot perform means testing on this set of data. Nonetheless, this study provided a starting point for gathering information on the effects of CLA isomers on levels of hepatic glucose-metabolizing enzymes.

Another point to consider is that while protein levels are important to examine, it is also important point to investigate the activation state of these proteins. Under conditions of low glucose, GK is sequestered in the nucleus by GKRP. When glucose concentrations within the hepatocyte rise, a conformational change in GKRP causes GK to be released and translocated to the cytosol for phosphorylation of glucose (Gregori et al., 2006). Therefore, determining the relative amounts of nuclear and cytosolic GK would provide an understanding of how CLA affects GK activity and the potential contribution of GK activity to improvement of oral glucose tolerance. Measuring the relative amounts of nuclear and cytosolic GK could be done with immunohistochemistry or Western blotting after subcellular fractionation.

Similarly, while protein levels of ChREBP are informative, activation of ChREBP is what allows it to participate in glucose metabolism. Activation of ChREBP involves two key phosphorylation steps that allow its nuclear translocation and DNA binding. Therefore, future studies should examine how dietary CLA affects these dephosphorylation steps for subsequent activation of ChREBP, as well as intracellular localization.

References

Dentin, R. Girard, J., & Postic, C. (2005). Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c): two key regulators of glucose metabolism and lipid synthesis in the liver. *Biochmie, 87*, 81-86.

Diakiw, R. P. (2005). Effects of specific conjugated linoleic acid (CLA) isomers on insulin resistance, and skeletal muscle AMP-activated protein kinase-alpha (AMPK-alpha) in *fa/fa* and lean Zucker rats [dissertation]. Winnipeg (Manitoba): University of Manitoba.

Gregori, C., Guillet-Deniau, I., Girard, J., Decaux, J. F., & Pichard, A. L. (2006). Insulin regulation of glucokinase gene expression: evidence against a role for sterol regulatory element binding protein 1 in primary hepatocytes. *FEBS Letters, 580*, 410-414.

Houseknecht, K. L., Vanden Heuvel, J. P., Moya-Camarena, S. Y., Portocarrero, C. P., Peck, L. W., Nickel, K. P., & Belury, M. A. (1998). Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker Diabetic Fatty *fa/fa* rat. *Biochemical and Biophysical Research Communications, 244*, 678-682.

Ishii, S., Iizuka, K., Miller, B. C., & Uyeda, K. (2004). Carbohydrate response element binding protein directly promotes lipogenic enzyme gene transcription. *Proceedings of the National Academy of Science U. S. A., 101*, 15597-15602.

Meyer, C., Dostou, J. M., Welle, S. L., & Gerich, J. E. (2002). Role of human liver, kidney, and skeletal muscle in postprandial glucose homeostasis. *American Journal of Physiology: Endocrinology and Metabolism, 282*, E419-E427.

Olson, A. L. & Pessin, J. E. (1996). Structure, function, and regulation of the mammalian facilitative glucose transporter gene family. *Annual Reviews in Nutrition, 16*, 235-256.

Ryder, J. W., Portocarrero, C. P., Song, X. M., Cui, L., Yu, M., Combatsiaris, T., Galuska, D., Bauman, D. E., Barbano, D. M., Charron, M. J., Zierath, J. R., & Houseknecht, K. L. (2001). Isomer-specific antidiabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. *Diabetes, 50*, 1149-1157.

Stoeckman, A. K., Ma, L., & Towle, H. C. (2004). Mlx is the functional heteromeric partner of the carbohydrate response element-binding protein in the glucose regulation of lipogenic enzyme genes. *Journal of Biological Chemistry, 279*, 15662-15669.

Van Schaftingen, E., Detheux, M., & Veiga da Cunha, M. (1994). Short-term control of glucokinase activity: role of a regulatory protein. *FASEB Journal, 8*, 414-419.

Weickert, M. O. & Pfeiffer, A. F. H. (2006). Signalling mechanisms linking hepatic glucose and lipid metabolism. Diabetologia, May 23, 2006 (E-pub ahead of print).

Yamashita, H., Takenoshita, M., Sakurai, M., Bruik, R. K., Henzel, W. J., Shillinglaw, W., Arnot, D., & Uyeda, K. (2001). A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. *Proceedings of the National Academy of Science U. S. A., 98*, 9116-9121.

APPENDIX 7

SCREENING FOR DIFFERENCES IN SELECTED INFLAMMATORY AND ENDOPLASMIC RETICULUM STRESS PROTEINS IN LEAN AND *fa/fa* ZUCKER RATS FED CLA ISOMERS

Introduction

It is now well-recognized that obesity is a state characterized by production of inflammatory and stress-related proteins. c-Jun NH_2-terminal kinase (JNK) is one such inflammatory mediator. Activated by stress or inflammatory stimuli, JNK contributes to insulin resistance by phosphorylation of IRS-1 on serine^{307}, reducing the amount of IRS-1^{tyr} phosphorylation for propagation of the insulin signal (Wellen & Hotamisligil, 2005; Aguirre et al., 2000). Of the three JNK isoforms, JNK-1 is highly expressed in skeletal muscle, liver, and adipose tissue, and its activity in these tissues is elevated in obesity, hepatic steatosis, and Type 2 diabetes (Hirosumi et al., 2002). Furthermore, blocking the expression and activity of JNK in livers of obese and insulin resistant mice results in increased systemic insulin sensitivity, improved glucose homeostasis and reduced IRS-1 serine phosphorylation (Nakatani et al., 2004; Bennet et al., 2003; Kaneto et al., 2004).

Although the association between cellular stress signaling and inflammatory pathways (such as those activated by JNK) and obesity is clear, the initial trigger of this stress is still poorly understood. Ozcan et al. (2004) propose the endoplasmic reticulum (ER) as a key sensor, integrator, and transmitter of the stress signals associated with obesity. The ER is the site of protein synthesis, folding, modification and transportation. Obesity, characterized by increased protein and lipid synthesis and lipid accumulation, causes the amount of "client traffic" reaching the ER to increase, initiating an adaptive response known as the

unfolded protein response (Ozcan et al., 2004). The main events during the unfolded protein response include transcriptional activation of chaperones to aid protein folding, selective reduction of new protein synthesis (allowing the ER to cope with existing proteins) and activation of proteosome-mediated protein degradation. Two such chaperone proteins are GRP78 and PERK. GRP78 is localized in the ER where it receives imported secretory proteins and is involved in the folding and translocation of new peptide chains (Schroder & Kaufman, 2005). PERK is an ER kinase that responds specifically to a stress signal generated in the lumen of the ER (Bertolotti et al., 2000). Activation of PERK induces a characteristic set of genes encoding ER chaperones and nuclear transcription factors that ultimately lead either to reduction of ER stress or to death (Mori, 2000).

In livers of *ob/ob* mice and high-fat fed mice, ER stress is present as evidenced by higher mRNA levels of GRP78, higher pPERK and higher JNK activity (Ozcan et al., 2004). Therefore, we hypothesized that the same results would be found in livers of *fa/fa* Zucker rats, a rat model for obesity and insulin resistance. We also hypothesized that t10,c12 CLA would reduce the elevated protein levels of inflammatory and ER-stress proteins. To investigate this hypothesis, we used a Multiscreen apparatus to examine differences in hepatic $pIRS^{tyr}$, JNK, pJNK, GRP78, PERK, and pPERK in lean and *fa/fa* Zucker rats as well as *fa/fa* Zucker rats fed CLA isomers. We also compared levels of hepatic LXRα, a nuclear receptor induced by insulin, which promotes lipogenesis and suppresses gluconeogenesis (Steffensen & Gustafsson, 2004).

Methods

Hepatic protein extracts from one control lean Zucker rat (ln CTL), one control *fa/fa* Zucker rat (fa CTL), one *fa/fa* rat from the fa 9-11 group (fa 9-11_ and one *fa/fa* rat from the fa 10-12 group (fa 10-12) were taken from Study 1. Hepatic protein extracts were subjected to SDS-PAGE and gel transfer as in Study 1 of this dissertation (see page 76), with the exception that a comb with one small well for the molecular mass marker and two large wells for samples was used to make the gel. Thus, only 2 samples at a time were loaded onto each gel. To compare the effect of genotype, one gel was loaded with the ln CTL sample and the fa CTL sample; to compare the effects of c9,t11 and t10,c12 CLA, one gel was loaded with the fa 9-11 sample and the fa 10-12 sample. A Mini Protean Multiscreen apparatus (Model #170-4017, BioRad, Richmond, CA) was used to simultaneously blot for multiple proteins on the same membrane. The Multiscreen apparatus has two main parts: membranes are positioned on the bottom tray, and the top part containing 20 "slots" clamps down onto the bottom tray. Each membrane is situated under a set of 20 slots. After blocking each membrane in 3% BSA for 1 hour, 100 µL of antibodies to the proteins of interest was pipetted into the corresponding slots (see Table A7-1 for a list of primary antibodies). The entire apparatus was then placed on a horizontal shaker for 1 hour. The primary antibodies were then aspirated out of the slots, and 100 µL of 1x TBST was pipetted into each slot. The 1x TBST was aspirated after 5 minutes, and 100 µL of 1x TBST was again pipetted into each slot. This washing

step was repeated for a total of 5 washes. One-hundred µL of secondary antibodies to the proteins of interest were then pipetted into the corresponding slots, and the apparatus was placed on the horizontal shaker for 1 hour, after which the secondary antibodies were aspirated. Five washing steps with 1x TBST were then performed. Chemiluminescence and autoragiography for detection of protein bands was performed as in Study 1 of this dissertation (see page 81).

Table A7-1 – Antibodies Used for Western Immunoblotting (Hepatic Inflammation and ER Stress Study)

Primary Antibody	Name	Source	Dilution[1]	Host	Secondary Antibody	Dilution[2]	Molecular Weight (kDa)
GRP78	GRP78 (N-20)	Santa Cruz Biotechnology, Inc., Cat. # sc-1050	1:1000	Goat	Anti-goat horseradish peroxidase[3]	1:10 000	78
pIRS-1	Phospho-IRS-1 (tyr941)	Oncogene Research Products, Cat. # GF1004	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	165
JNK	SAPK/JNK	Cell Signaling, Cat. # 9252	1:1000	Rabbit	Anti-rabbit horseradish peroxidase	1:10 000	46 (JNK 1) 54 (JNK 2&3)
pJNK	Anti-ACTIVE JNK pAb	Promega, Cat. # V7931/2	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	46 (JNK 1) 54 (JNK 2&3)
LXRα	LXRα (H-144)	Santa Cruz Biotechnology, Cat. # sc-13068	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	50
PERK	PERK (H-300)	Santa Cruz Biotechnology, Inc., Cat. # sc-13073	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	170
pPERK	Phospho-PERK (Thr980)	Cell Signaling, Cat. # 3191	1:1000	Rabbit	Anti-rabbit horseradish peroxidase[3]	1:10 000	170

[1] Primary antibodies diluted with 3% BSA-TBST.
[2] Secondary antibodies diluted with 1% BSA-TBST.
[3] Membranes incubated in secondary antibody for 1 hour.

Results and Discussion

Multiscreen blots were examined visually for differences in protein levels. When comparing the lean and *fa/fa* Zucker rat, the only protein that could be detected was JNK (Figure A7-1). Visually, it appeared that JNK protein level in liver of the fa CTL rat was higher than the ln CTL. This result is consistent with other studies that have shown higher JNK protein levels in livers of obese and insulin resistant animals (Ozcan et al., 2004).

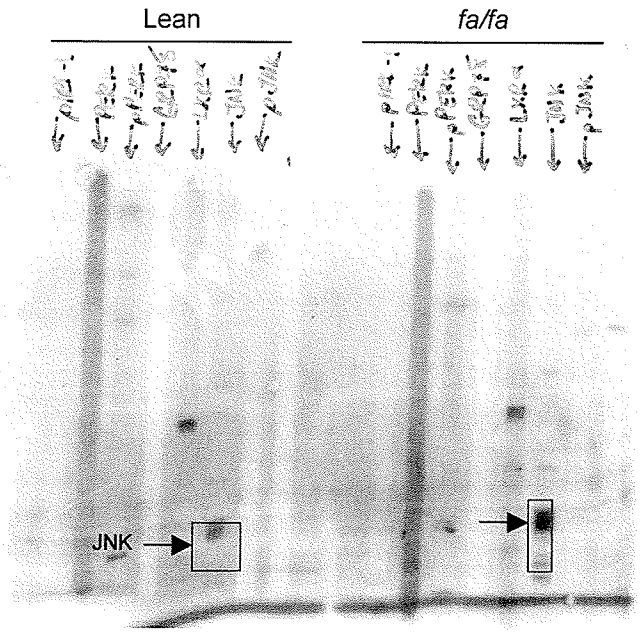

Figure A7-1. Multiscreen on lean Zucker rat and *fa/fa* Zucker rat.

More proteins were detectable on the blot comparing the fa 10-12 rat to the fa CTL rat (Figure A7-2). Hepatic levels of JNK and pJNK did not appear to be different between the two rats. LXRα levels appeared higher in the fa 10-12 rat than the fa CTL rat. Levels of pPERK appeared similar in both groups; however, levels of PERK appeared lower in the fa 10-12 rat than the fa CTL rat. This may have been due to differences in film exposure, however, the entire PERK lane of the fa CTL rat is darker than the PERK lane of the fa 10-12 rat.

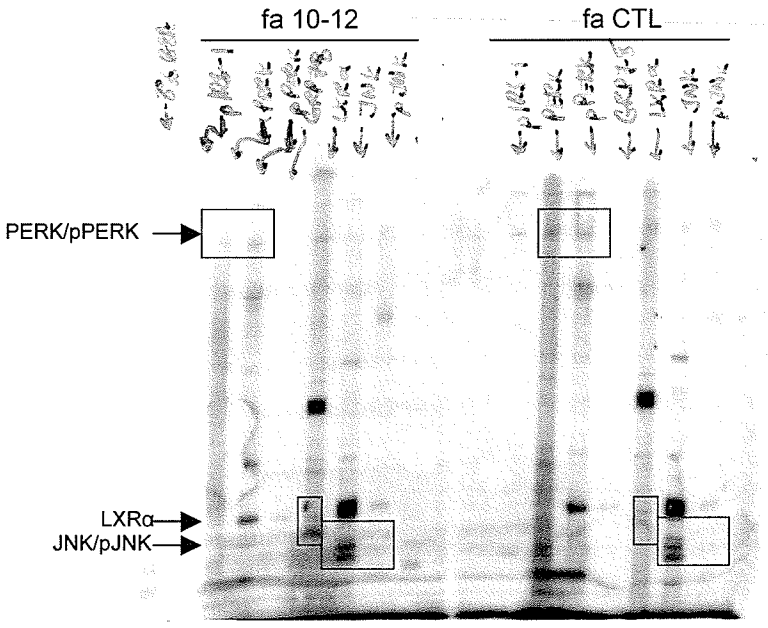

Figure A7-2. Multiscreen on fa 10-12 Zucker rat and fa CTL Zucker rat.

When examining the blot comparing the fa 9-11 and fa 10-12 rats, only JNK and pJNK could be detected (Figure A7-3). Hepatic levels of JNK appeared slightly higher in the fa 9-11 rat than the fa 10-12 rat. There appeared to be no difference in pJNK levels between the two rats.

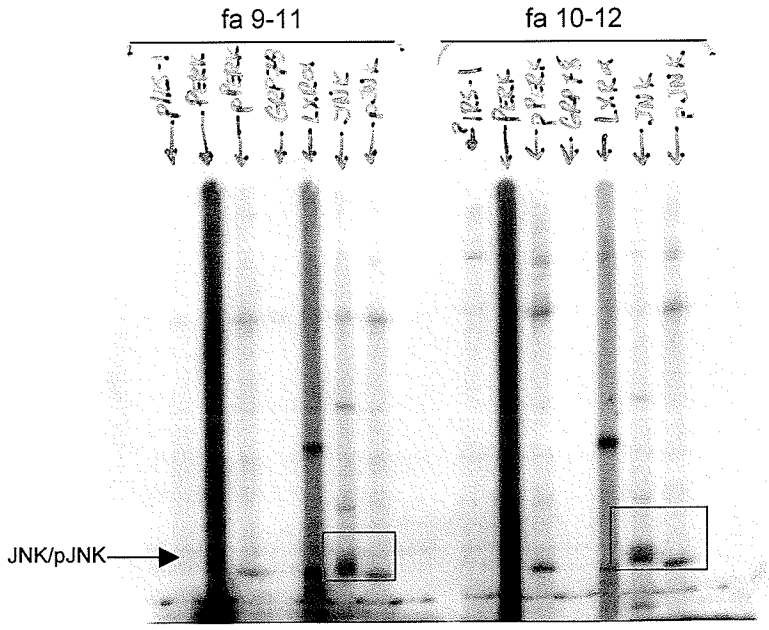

Figure A7-3. Multiscreen on fa 9-11 Zucker rat and fa 10-12 Zucker rat.

In this small preliminary study, we used a Multiscreen apparatus to assess changes in inflammatory and ER stress proteins based on genotype and dietary CLA feeding. While this apparatus provides the advantage of being able to blot for a number antibodies simultaneously, it also has disadvantages. Each antibody requires a different exposure time; therefore, some of the lanes become

overexposed while other remain underexposed, resulting in the need for multiple exposure times of the same membrane. In addition, the nature of the apparatus allows analysis of only 2 samples; therefore, results from these blots must be confirmed with further analysis. Regardless, this apparatus provides a quick method to screen for large changes in expression of multiple proteins between 2 samples.

References

Aguirre, V., Uchida, T., Yenush, L., Davis, R., & White, M. F. (2000). The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *Journal of Biological Chemistry, 275*, 9047-9054.

Bennett, B. L., Satoh, Y., & Lewis, A. J. JNK: a new therapeutic target for diabetes. *Current Opinions in Pharmacology, 3*, 420-425.

Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., & Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nature Cell Biology, 2*, 326-332.

Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., Karin., M., & Hotamisligil, G. S. (2002). A central role for JNK in obesity and insulin resistance. *Nature, 420*, 33-336.

Kaneto, H., Nakatani, Y., Miyatsuka, T., Kawamori, D., Matsuoka, T. A., Matsuhisa, M., Kajimoto, Y., Ichijo, H., Yamasaki, Y., & Hori, M. Possible novel therapy for diabetes with cell-permeable JNK-inhibitory peptide. *Nature Medicine, 10*, 1128-1132.

Mori, K. (2000). Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell 101,* 451-454.

Nakatani, Y., Kaneto., H., Kawamori, D., Hatazaki, M., Miyatsuka, T., Matsuoka, T. A., Kajimoto, Y., Matsuhisa, M., Yamasaki, Y., & Hori, M. (2004). Modulation of the JNK pathway in liver affects insulin resistance status. *Journal of Biological Chemistry, 279*, 45803-45809.

Ozcan, U., Cao, Q., Yilmaz, E., Lee, A. H., Iwakoshi, N. N., Ozdelen, E., Tuncman, G., Gorgun, C., Glimcher, L. H., & Hotamisligil, G. S. (2004). Endoplasmic reticulum stress links obesity, insulin action, and Type 2 diabetes. *Science, 306*, 457-461.

Schroder, M. & Kaufman, R. J. (2005). The mammalian unfolded protein response. *Annual Reviews in Biochemistry, 74*, 739-89.

Steffensen, K.R. & Gustafsson, J. A. (2004). Putative metabolic effects of the liver X receptor (LXR). *Diabetes, 53* (Suppl 1), S36-S42.

Wellen, K. E. & Hotamisligil, G. S. (2005). Inflammation, stress, and diabetes. *Journal of Clinical Investigation, 115*, 1111-1119.