The Effect of Conjugated Linoleic Acid (CLA) Isomers on Hepatic Lipid Droplets and Lipid Droplet Proteins in *fa/fa* Zucker Rats

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

Master of Science

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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The Effect of Conjugated Linoleic Acid (CLA) Isomers on Hepatic Lipid Droplets and Lipid Droplet Proteins in *fa/fa* Zucker Rats

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ABSTRACT

Type 2 diabetes and nonalcoholic fatty liver disease (NAFLD) are some of the consequences of obesity. It is hypothesized that conjugated linoleic acid (CLA) can be used as a therapeutic agent to treat NAFLD in *fa/fa* Zucker rats through its ability to reduce lipid droplet formation and decrease the cellular level of associated lipid droplet proteins (adipophilin and perilipin).

The first objective was to determine the effects of CLA isomers (0.4% *cis*-9,*trans*-11 and 0.4% *trans*-10,*cis*-12) on the number and size of lipid droplets and the lipid droplet proteins adipophilin and perilipin in 17 week old *fa/fa* Zucker rats after an 8 week dietary intervention. The second objective was to determine the effects of peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR) and farnesoid X receptor (FXR) agonists on adipophilin and perilipin in a cell culture model of lipid accumulation using hepatic H4IIE cells.

Treatment with the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers reduced the lipid droplet area compared to control group but the number of lipid droplets was similar in all *fa/fa* rats regardless of dietary treatment. The *trans*-10,*cis*-12 CLA isomer significantly decreased hepatic adipophilin but not perilipin protein levels in the *fa/fa* rats. The cell culture study showed that cells treated with the PPARγ agonist rosiglitazone and the PPARα agonist WY14643 had lower amounts of adipophilin protein compared to the other treatments.

In conclusion, the anti-steatotic effects of *trans*-10,*cis*-12 CLA in older *fa/fa* Zucker rats with established obesity and metabolic syndrome were associated with

reduced levels of the lipid droplet protein adipophilin as well as smaller lipid droplet area in the liver. The cell culture study indicated that adipophilin may be regulated by PPARα and PPARγ, and reductions in lipid accumulation and adipophilin levels in H4IIE cells are mediated by PPARγ and PPARα, but not LXR or FXR.

ACKNOWLEDGEMENTS

I would like to acknowledge my co- supervisors, Dr. Carla Taylor and Dr. Peter Zahradka, for their patience, support and guidance throughout my project. They were very understanding, helpful and great mentors. Thank you for allowing me to be part of your research group. Thank you to Dr. Miyoung Suh and Dr. Jon McGavock for being a part of my Committee. Their wisdom and input is greatly appreciated.

Thank you to Dr. Kevin Coombs and Dr. James D. House for helping me to achieve my goals.

I would like to thank Leslee Tworek for her time and help with lab-related work. Also, I would like to thank Heather Blewett for being very helpful and teaching me new techniques. Thank you to all my fellow graduate students in our lab.

Thank you to Pat Parish and Sue Zettler for administrative support.

Thank you to Dairy Farmers of Canada and the Natural Science and Engineering Research Council of Canada for providing funding for this project.

A special sincere thank you to my Mother Azarmidokht Nourainejad for being there for me. Lastly, I would like to thank my family, Afsaneh, Atta, Sohrob and Sara and all my friends, especially Fereshteh, for their patience, support, love and time.

DEDICATION

In memory of my father Mr. Seyedaliasghar Kazem Moosavi, the man who inspired me all of my life and my mother Azarmidokht Nourainejad.

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ABBREVIATIONS

9-11	*cis*-9,*trans*-11 CLA diet
10-12	*trans*-10, *cis*-12 CLA diet
ACO	acyl-CoA oxidase
ALT	alanine aminotransferase
ANOVA	analysis of variance
BA	bile acid
BMI	body mass index
BSA	bovine serum albumin
MEM Alpha	Minimum Essential Medium Eagle, Alpha
c9,t11	*cis*-9, *trans*-11
CIC	citrate carrier
CLA	conjugated linoleic acid
CTL	control diet
DBD	DNA-binding domain
DM-2	diabetes mellitus-type 2
ddH_2O	distilled, deionized water
kDa	kilodalton
MUFA	monounsaturated fatty acid
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
LXR	liver-X-activated receptor
LBD	ligand binding domain
OA	oleic acid

ob/ob	obesity gene
OCT	optimal cutting temperature compound
OLETF	Otsuka Long-Evans Tokushima fatty
PBS	phosphate-buffered saline
PO	pioglitazone
PPAR	peroxisome proliferators – activated receptors
PVDF	polyvinylidene
RE	response element
RO	rosiglitazone
RXR	retinoic acid receptor
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SCD-1	stearoyl-CoA desaturase-1
SEM	standard error of mean
SFA	saturated fatty acid
SREBP	sterol regulator element-binding protein
TAG	triacylglycerol
TBS	Tris buffered saline
TBST	Tris-buffered saline with Tween-20
TEMED	N, N, N', N'-Tetramethylethylenediamin
TNF-α	tumor necrosis factor-α
TZD	thiazolidinediones
t10,c12	*trans*-10, *cis* -12
VLDL	very-low density lipoprotein

WHO	World Health Organization
wt/wt	weight by weight
ZDF	Zucker diabetic fatty

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LITERATURE REVIEW

Excessive body weight is one of the most important risk factors for various diseases worldwide. Cardiovascular disease, type 2 diabetes, cancer, liver disease and other complications are some of the consequences of obesity. Today, there is a growing interest in finding molecules including dietary components that have beneficial effects on obesity and its complications. The purpose of the following literature review is to investigate current knowledge about conjugated linoleic acid (CLA) as a potential therapeutic agent for the management of certain characteristics of metabolic syndrome, especially obesity and non-alcoholic fatty liver disease (NAFLD). CLA is a mixture of positional and geometric isomers of linoleic acid, and it has attracted considerable attention because of its potentially beneficial biological effects both *in vitro* and *in vivo*. NAFLD is emerging as an important complication of obesity.

Nonalcoholic Fatty Liver Disease (NAFLD)

Nonalcoholic fatty liver disease (NAFLD) refers to a wide range of liver diseases including simple fatty liver (steatosis), nonalcoholic steatohepatitis (NASH), cirrhosis, and end-stage liver disease (Angula, 2005). It is emerging as the most common liver disease in industrialized countries. Confirmation of the disease can be achieved by imaging studies and staging the disease requires a liver biopsy. Treatment is based on

weight loss and exercise, although insulin-sensitizing agents, antioxidants and medications are promising (Papandreou, 2007). The risk of NASH progressing to cirrhosis is approximately 20% in 10-20 years (Younossi et al., 2002). NAFLD is one of the most prevalent forms of the liver diseases. The prevalence of NAFLD is 20% and the prevalence of NASH is 2-3% in general population in the United States (Younossi et al., 2002).

Hepatic steatosis is the accumulation of triacylglycerols (TAG) in the liver cell and is diagnosed when lipid content in the liver exceeds 5-10% by weight (Reddy et al., 2006). This could be accompanied by clinical signs such as insulin resistance and liver malfunction. Numerous conditions contribute to NAFLD, but the exact etiology is still unknown. Obesity and insulin resistance are associated with the increased prevalence of hepatic steatosis. Hepatic steatosis is a common feature of metabolic syndrome. Currently 75% of persons with obesity and diabetes have some degree of NAFLD (Purushotham et al., 2007). The proportion of obese children tripled in last 25 years, and currently, two out of three adults in Canada are overweight or obese (Health Canada, 2009a).

Type 2 Diabetes (DM-2)

Type 2 diabetes (DM-2) is an increasingly common cause of morbidity and mortality in the world. It is reported to affect 3-5% of the population worldwide and 10-60% of individuals over 60 years of age (Leonard et al., 2005). In 2005-2006, Canada had approximately 2 million people diagnosed with diabetes; this represents about 1 in 17 Canadians. The number of people with diagnosed diabetes continues to grow. Two

hundred thousand new cases of diabetes were diagnosed in 2005-2006; however, it is now recognized that a large number of individuals are not aware they have this disease (Public Health Agency of Canada, 2009).

DM-2 occurs when target cells cannot take up glucose in sufficient amounts and blood glucose become elevated. It is characterized by a reduced sensitivity to the effects of insulin and eventually results in impaired β-cell function (Upton et al., 1998). Many people with DM-2 also have metabolic syndrome. There is a decreased ability of insulin to stimulate the use and storage of glucose which results in hyperglycemia and diabetes.

Obesity is one of the major risk factors for DM-2. Obesity is defined as a body mass index (BMI) of greater than 30 kg/m^2 (Health Canada, 2009b). BMI is a simple index of weight-for-height that is commonly used in classifying overweight and obesity in adult populations and individuals. It is defined as the weight in kilograms divided by the square of the height in meters. Obesity and DM-2 are also risk factors for NAFLD. In obese animals, there is an increase in glucose conversion to lipid in all tissues, including the liver, due to an increase in lipogenesis. Insulin-stimulated glucose uptake is also significantly less in skeletal muscle (Leonard et al., 2005).

Insulin resistance in DM-2 may be due to low numbers or lower activity of insulin receptors or loss of post receptor function (Sugden et al., 2008). The Zucker Diabetic Fatty (ZDF) rat is a commonly used animal model of DM-2. These rats were derived from the inbreeding of hyperglycemic Zucker obese rats. ZDF rats have a mutation in the extracellular domain of the leptin receptor. This mutation impairs the appetite suppressing effects of leptin and it also has thermogenic effects. These animals exhibit

obesity, hyperphagia, polyuria, polydipsia, and have insulin resistance. Hyperglycemia develops when these animals are between 7-12 weeks (Leonard et al., 2005).

Metabolic Syndrome

NAFLD is associated with dyslipidemia, obesity, and insulin resistance, which are the main features of metabolic syndrome. Metabolic syndrome is a major public health concern. Metabolic syndrome is a cluster of characteristics including abdominal obesity, atherogenic dyslipidemia, raised blood pressure, insulin resistance, a prothrombotic state, and a pro-inflammatory state (National Cholesterol Education Program, 2009). Overweight/obesity, physical inactivity and genetic factors are risk factors for the metabolic syndrome.

The therapeutic options for managing individuals with metabolic syndrome usually target the major symptoms and are designed to reduce body weight and hypertension, and improve lipid and glucose irregularities. The beneficial effects of CLA, such as body fat reduction in mice and liver lipid lowering in rats, have led to suggestions that CLA could be used for the treatment of the metabolic syndrome.

The *fa/fa* Zucker rat is a good model of obesity and the metabolic syndrome. The *fa/fa* Zucker rat develops metabolic and hormonal disorders that share many features with human obesity. The *fa/fa* Zucker rats have hyperphagia due to a missense mutation on the leptin receptor gene; they become obese, hyperinsulinemic, hypertensive and have NAFLD (Taylor and Zahradka, 2004).

Conjugated Linoleic Acid (CLA)

Conjugated linoleic acid (CLA) refers to a mixture of naturally occurring positional and geometric isomers of conjugated dienoic derivatives of linoleic acid (Wang et al., 2004). CLA is produced by bacteria via biohydrogenation of linoleic and α-linoleic acid in the ruminant intestine (Granlund et al., 2003; Ferramosca et al, 2006). The *cis*-9, *trans*-11 (c9,t11) CLA isomer is the major isomer in ruminant meats (beef, dairy, sheep, and goat), ruminant milk, dairy products (yogurt, cheese, butter) and human breast milk. The CLA content of cheese ranges between 3.59 to 7.96 mg CLA/g lipid (Lin et al., 1995). Fermented dairy products contained 3.82 to 4.66 mg CLA/g lipid. Fluid milk contains 3.38-6.39 mg CLA/g lipid and its CLA content changes based on seasonal and geographical situation (Banni, et al., 1996). CLA content in cow's milk is higher in spring and summer due to the presence of more polyunsaturated fatty acids in their diet (Kraft et al., 2003). CLA in milk or meat is stable during cooking and storing. Human CLA intake is varies between 151 mg for women and 212 mg for men for those not consuming CLA commercial preparations, however, these amounts are variable based on the individual's diet.

The c9,t11 isomer accounts for more than 90% of the total CLA intake from dietary sources (Silvereira et al., 2007). Many feeding studies use synthetically produced CLA which contains a mixture of c9,t11 and t10,c12 isomers. These isomers of linoleic acid have been shown to have anticarcinogenic, antiatherogenic, antidiabetes, and antiobesity properties (Granlund et al., 2003). The rapid increase in the prevalence of obesity has attracted considerable attention to CLA because of its beneficial effects to

decrease body fat mass while enhancing lean body mass in some species (Tsuboyama-Kasaoka et al., 2000).

CLA is able to reduce adiposity by affecting energy and lipid metabolism (Leaver et al., 2006). CLA reduces body fat mass in mice, rats, hamsters and pigs; however, CLA causes liver enlargement and increases tissue lipid content in mice (Takahashi et al., 2003). Results in humans have been inconsistent. In overweight/obese subjects, 4 of 7 studies observed a reduction in body fat whereas the others reported no change (Lee et al., 1994). The different results with respect to species and age-related responses may be due to factors such as the treatment dose, duration and background diet.

Studies have demonstrated that the c10,t12 isomer of CLA is most likely responsible for reducing body fat (Ferramosca et al., 2006). CLA administration in mice decreases body fat mass, but it produces lipid accumulation in the liver. Although CLA reduces body fat, the enlargement of the liver has raised concerns about its safety. In C57BL/6J mice, dietary supplementation with 1% c9,t11 and t10,c12 CLA for 1 week caused massive fatty liver (Wang et al., 2004). Wang et al., (2005) investigated the effects of short-term feeding with 2% CLA for 1 week on adipose tissue weights, liver weight and hepatic lipid metabolism in C57BL/6J mice. In this study, short-term feeding of CLA resulted in lipodystrophy in C57BL/6J mice without inducing adverse effects in the liver.

Other researchers have investigated effects of CLA on hepatic steatosis and adiposity in rat models. Purushothem et al., (2007) investigated the effect of a CLA mixture (39.2% c9,t11 and 38.5% t10,c12) on liver lipid in a rodent model for hepatic steatosis which is resistant to the adipose-lowering effects of CLA. Obesity in these rats

was induced by diet and without any genetic manipulation. After feeding the Wistar rats with a high fat diet (20% fat) for four weeks to induce obesity and hepatic steatosis, the diet was switched to a low fat diet (6.5% fat) with either soybean oil or soybean oil and the CLA mixture for an additional four weeks. The rats fed CLA during the low fat phase had significantly decreased hepatic lipid accumulation without altering adipose mass. CLA significantly increased the mRNA level of PPAR-α and increased peroxisomal oxidation in the liver (Purushotham et al., 2007).

Nagao et al. (2005) tested whether dietary CLA protects 6 week old male *fa/fa* Zucker rats from hepatic injury. The CLA group was fed a 1% CLA mixture (46% c9,t11, 47.3% t10,c12) and compared with a control group in which CLA was replaced with 1% linoleic acid. After 8 weeks of feeding, hepatomegaly and hepatic TAG accumulation were reduced in the CLA-fed Zucker rats compared with the control group. It was suggested that hepatic steatosis resulted from increased hepatic β-oxidation. In the CLA diet group, the activity of carnitine palmitoyltransferase (the key enzyme of fatty acid β-oxidation) was enhanced and microsomal TAG transfer protein (a factor for lipoprotein secretion) was improved. They speculated that an elevation in plasma adiponectin and a reduction of liver tumor necrosis factor-α (TNF-α) mRNA in the CLA-fed *fa/fa* Zucker rats may be associated with the improvement in hepatic steatosis. CLA acts as an inducer of adiponectin and adiponectin is primarily secreted from adipose tissue in rodent and human. Adiponectin has been reported to alleviate alcohol or obesity-induced hepatomegaly, and hepatic steatosis in mice (Xu et al., 2003). In humans, adiponectin has a protective role against NAFLD (Bajaj et al., 2004; Nagao et al., 2005).

A study by Wendel and Belury (2006) investigated the effects of 2 week diet supplementation with either 1.5% CLA or 0.2% troglitazone (TZD) in 6 week old male ZDF rats. Thiazolidenediones such as troglitazone are insulin sensitizing agents that can lower fasting blood glucose and reduce hepatic TAG levels and are used in DM-2 therapy. Thiazolidenediones function as high affinity ligands for nuclear receptor peroxisome proliferator activated receptor-gamma (PPARγ). PPARγ stimulates the differentiation, proliferation, and lipid accumulation of adipocytes, and promotes lipid accumulation in adipose tissue, but prevents lipid accumulation in peripheral tissues such as liver. The study by Wendel and Belury (2006) compared CLA and the thiazolidenedione troglitazone because many of the antidiabetic effects of CLA and thiazolidinediones are similar. Lipid accumulation and body composition of both lean and ZDF rats were measured after 2 weeks of feeding. ZDF with control diet developed significant hepatic steatosis while the hepatic TAG level in both CLA-fed and TZD-fed ZDF rats were similar to those of lean rats. Also, the ratio of 16:1/16:0 and 18:1/18:0 fatty acids were reduced in the liver of ZDF rats fed either the CLA or TZD diet. The 16:1/16:0 and 18:1/18:0 ratio is a marker for stearoyl-CoA desaturase-1 (SCD-1) activity. The CLA diet reduced adipose mass, however, TZD had no effect. The results showed that both TZD and CLA improved hepatic steatosis and fatty acid composition in ZDF rats, and suggests that changes in hepatic lipid composition maybe associated with a reduction in SCD-1.

Mechanism(s) of Action for CLA in Hepatic Steatosis

In an effort to understand the mechanism by which CLA modulates hepatic steatosis, different hypotheses have been suggested. One of the suggestions is that CLA affects the activity of the nuclear receptor family of transcription factors, which includes PPARα, PPARγ, liver X receptor-alpha (LXRα) and Farnesoid X receptors (FXR) (Taylor and Zahradka, 2004). These transcription factors will be discussed in more detail in another section.

CLA incorporation into cellular lipid alters fatty acid composition and can inhibit lipogenesis and TAG esterification by disrupting the fatty acid desaturation process. SCD-1 also known Δ9 desaturase is one of key desaturation enzymes in lipogenesis. Desaturation of saturated fatty acids (SFA) is necessary to produce TAGs. It has been suggested that CLA reduces SCD-1 mRNA expression and activity, and inhibits *de novo* fatty acid and TAG synthesis (Choi et al., 2000). This is supported by observations that CLA treatment increases the saturated (SFA):monounsaturated fatty acid (MUFA) ratio (Purushotham et al., 2007).

Other hypotheses focus on changes in energy metabolism due to CLA (Sakona et al., 1999). In mice, CLA increases energy expenditure, fatty acid oxidation and lipolysis, and this could alter the amount of lipid in the liver. The effects of CLA on energy expenditure and lipid metabolism depend on isomer type, dose and duration of the diet as well as the metabolic status and species of the experimental subjects. The t10, c12 CLA isomer is more efficiently oxidized than other isomers (Evans et al., 2002). In mice, it has been demonstrated that CLA increases the activity of important enzymes associated with hepatic lipogenesis. Hepatic lipogenesis occurs partly in the mitochondrial matrix

and partly in the cytosol. These two different compartments are connected with a mitochondrial transport protein called citrate carrier (CIC) which has an important role in intermediary metabolism. The CIC activity is down-regulated during starvation, while in mice fed diet enriched with a mixture of c9,t11 and t10,c12 for 16 weeks, CIC activity was increased along with an elevation of hepatic TAGs (Ferramosca et al., 2006).

Our laboratory has begun to study the effects of CLA on lipid droplet proteins as a new mechanism for understanding effects of CLA on hepatic steatosis (Stringer et al., 2009). In vertebrate animals, most of the energy is stored in the lipid droplets, primarily in adipocytes. Other tissues such as liver also have lipid droplets. The next section will describe the proteins that control lipid droplet formation and hydrolysis, lipid traffic in cells and the regulation of whole body energy metabolism.

Lipid Droplets and Lipid Droplet Proteins

Adipocytes hold the body energy reserves as TAG in lipid droplets. Lipid droplets can be as large as 100 μm and they have a hydrophobic TAG core, a phospholipid and cholesterol monolayer and embedded proteins (Brasaemle et al., 2004). Obesity increases TAG storage in adipose depots. When this TAG storage overflows, free fatty acids are released and accumulate as TAGs in non-adipose tissues, a process that is called steatosis (Wolins et al., 2006). These non-adipose tissues are not adapted for excess TAG storage and this causes complications such as dyslipidemia, insulin resistance, β-cell failure and hypertension.

The majority of TAG in mammalian cells is in droplets coated with one or more members of the perilipin-adipophilin-TIP47 (PAT) family (Bickel et al., 2009). Perilipin

and adipophilin are the most abundant proteins on these lipid droplets. The ideal location for perilipin and adipophilin to able to regulate the lipid pool is on the surface of lipid droplets. These proteins play a critical role in both TAG synthesis and hydrolysis. Mice lacking perilipin cannot accumulate large adipose TAG stores and hormone regulated lipolysis is muted (Wolins et al., 2006). This shows that perilipin inhibits lipolysis and promotes TAG storage in fed animals. Perilipin is largely limited to adipose tissue and steroidgenic cells, whereas adipophilin (adipose differentiation related protein, ADRP) has a broad tissue distribution. The three protein isoforms of perilipin are named perilipin A, B and C and they are the result of alternative splicing of a single gene transcript (Brasamle et al., 2004).

The mechanism of how perilipin controls TAG stores is still under investigation. Adipophilin in non-adipocytes inhibits TAG hydrolysis, the same role as perilipin in adipocytes. In cultured cells (murine fibroblast cell lines, 3T3-L1, NIH-3T3 and Swiss-3T3), the over-expression of perilipin or adipophilin increases TAG storage in the cell (Wolins et al., 2006 & Imamura, et al., 2002). Adipophilin is less protective against TAG hydrolysis than perilipin (Wilson et al., 2006). The stimulation of lipolysis by β-adrenergic agonists triggers phosphorylation of perilipin and causes translocation of hormone sensitive lipase to the surface of lipid droplets resulting in gradual fragmentation and dispersion of lipid droplets.

Adipophilin and perilipin are proteins which associate with the lipid droplets of adipocytes and their function is to control lipolysis. Perilipin protects TAGs from lipases and helps to promote TAG storage. The mechanism by which perilipin controls adipocyte lipolysis is based on the nutritional status. In the fed state, phosphorylation of perilipin

leads to the formation of a barrier at the surface of the lipid droplet which restricts the access of lipase to TAG. This effect reduces the rate of catalysis. Perilipin also acts as a scaffold at the surface of lipid droplets and in this way acts as an organizer for enzyme metabolism. In the fed state, proteins bind to this scaffold, stabilize the lipid droplets and protect them from lipolysis. Upon stimulation of lipolysis, lipid droplet remodeling is stimulated and this promotes motility of micro droplets (Brasaemle et al., 2008).

CLA, Lipid Droplet Proteins, and Hepatic Steatosis

It is possible that agents with the ability to reduce lipid droplet protein expression could inhibit hepatic steatosis. Our laboratory has reported that dietary CLA supplementation can reduce the hepatic accumulation of lipids in *fa/fa* Zucker rats (Noto et al., 2006). More recently, Stringer et al. (2009) examined the effects of CLA isomers on lipid droplet proteins in this model. Six-week old male *fa/fa* Zucker rats were fed four different diets (0% CLA, 0.4% c9,t11 CLA, 0.4% t10,c12 CLA, and a mixture of 0.4% c9,t11 CLA and 0.4% t10,c12 CLA) for 8 weeks. The results of this study showed that liver lipid concentration decreased in the t10,c12 CLA and CLA mixture groups, and this was associated with improvemed liver function as determined by alanine aminotransferase (ALT). Liver adipophilin levels were significantly lower in the t10,c12 group compared to the control group, but there were no changes observed in perilipin among the groups (Stringer et al., 2009). To my knowledge, this is the first study to show the effect of CLA on lipid droplet proteins in a model of hepatic steatosis.

The *fa/fa* Zucker Rat as a Model for Hepatic Steatosis

There are several genetic rodent models for obesity, metabolic syndrome and diabetes. In mice, the obesity and diabetes syndromes are caused by two single autosomal recessive mutations, obese (*ob*) and diabetes (*db*). The *ob/ob* mice are unable to produce satiety factor (leptin), while *db/db* mice are resistant to it due to a mutation in the leptin receptor. The *ob* gene encodes leptin which is expressed specifically in the adipose tissue while the leptin receptor is expressed in several tissues. Leptin acts as a sensor of fat mass.

The rat gene for fatty (*fa*) is a homologue of the mouse *db* gene, and *fa/fa* rats have a mutation in the extracellular domain of the leptin receptor (Takaya etal.1996). The *fa/fa* rats accumulate adipose mass soon after birth and obesity becomes apparent as early as 3 weeks of age (Truet et al., 2000). The Zucker rats develop a syndrome that shares many features with human obesity such as hyperphagia, hyperinsulinemia (by 4-5 weeks), hypertension (at six weeks) and NAFLD (Nagao et al., 2005). Insulin and leptin resistance develop in parallel and both could contribute to hepatic lipid accumulation. However, a study by Fishman et al. (2007) provides evidence that failure of leptin action is the primary determinant for hepatic steatosis. Thus, in the fa/fa Zucker rat, hepatic steatosis could be due to leptin resistance since leptin plays an important role in regulating fat metabolism and fat distribution. Unlike ZDF rats, they do not develop hyperglycemia and diabetes. In *fa/fa* rats, hyperinsulinemia is present and indicates an increase insulin levels in the blood due to insulin resistance. The *fa/fa* Zucker rat is considered a model of the prediabetic state with hepatic steatosis (Nagao et al., 2003a).