ROLE OF BIOACTIVE COMPOUNDS IN THE REGULATION OF INSULIN SENSITIVITY

DISSERTATION

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the Degree Doctor of Philosophy in the Graduate
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Hepatic steatosis and insulin resistance are often associated with obesity resulting in the development of overt type 2 diabetes. Dietary agents have important implications in the treatment of chronic diseases related to obesity and type 2 diabetes. In the first part of this dissertation project, the role of the potent weight loss agent, conjugated linoleic acid (CLA) in hepatic insulin resistance was investigated. My first objective was to determine the effectiveness of CLA in the attenuation of hepatic steatosis in a diet-induced model of obesity. Adult Wistar rats were fed a high fat diet (20% fat) for four weeks to induce obesity and hepatic lipid accumulation. Then, rats were randomly assigned to low fat (6.5% fat) experimental diets containing either 6.5% soybean oil (CON diet) or 1.5% CLA triglyceride mix plus 5% soybean oil (CLA diet) for an additional four weeks. CLA significantly decreased hepatic triglycerides (TG) compared to the CON diet. This CLA mediated difference was associated with increases in the mRNA levels of genes required for lipid oxidation such as acetyl-CoA oxidase (AOX) and peroxisome proliferator-activated receptor (PPAR-α) and significantly decreased expression of genes indicative of lipogenesis, fatty acid synthase, stearoyl CoA desaturase-1 (SCD-1) and sterol regulatory element binding protein-1a. Furthermore, hepatic SCD-1 index, a marker for SCD-1 activity, was also significantly decreased in both neutral and phospholipid fractions in rats supplemented with CLA.
The effects of CLA were observed in the absence of decreases in adiposity, adipokines, body weight and food intake in Wistar rats suggesting that CLA is protective against hepatic TG accumulation in high fat-fed rats by modulating hepatic lipid metabolism.

In marked contrast to the above findings, supplementation with CLA is associated with the development of hepatic steatosis and insulin resistance in mice. These effects of CLA occur along with rapid ablation of adipose tissue and significant and rapid depletion of insulin sensitizing adipokines, leptin and adiponectin. Therefore, my second objective was to determine the role of adipokines in CLA mediated insulin resistance in mice. Male C57BL/6 mice were fed a low fat (6.5% total fat) diet supplemented with 1.5% CLA for four weeks followed by switching mice to a diet without CLA for an additional four weeks. Four weeks of CLA supplementation significantly decreased body fat, induced hepatic steatosis and insulin resistance in mice. Furthermore, these changes were associated with significant depletion of adiponectin, but not leptin. Removing CLA from the diet attenuated both hepatic steatosis and insulin resistance along with significant increases in adiponectin. To further investigate the role of adiponectin in CLA mediated insulin resistance, male \textit{ob/ob} mice that lack functional leptin, were fed either a control diet (6.5% soybean oil) or CLA diet (1.5% CLA plus 5% soybean oil) containing diet as part of a low fat (6.5%) diet and an adiponectin enhancer and insulin sensitizer rosiglitazone (ROSI) was administered for two weeks. In the absence of leptin, maintenance of adiponectin alone prevented the development of insulin resistance in \textit{ob/ob} mice fed dietary CLA. In addition, maintenance of adiponectin significantly decreased hepatic TG in mice fed CLA (CLA-ROSI) compared to mice fed the CON diet.
with or without ROSI (CON-PBS, CON-ROSI). Taken together, these studies demonstrate that depletion of adiponectin causes insulin resistance in mice fed diet supplemented with CLA. When adiponectin levels were maintained (by removing CLA from the diet or by administering ROSI), hepatic steatosis and insulin resistance were significantly attenuated.

Because increased hepatic glucose production (HGP) contributes to elevated fasting glucose levels in type 2 diabetes, my third objective was to determine the effectiveness and elucidate the mechanisms by which dietary bioactive compounds, especially the citrus fruit flavonoid naringenin, exert glucose lowering effects in cultured hepatocytes. The aglycone naringenin significantly suppressed glucose production from Fao hepatoma cells similar to insulin and metformin. In contrast, the glucoside naringin had no effect on glucose production from Fao cells suggesting that the aglycone form is likely the active compound. Furthermore, incubating Fao cells with naringenin in the presence of an inhibitor of PI3kinase had no effect on the ability of naringenin to suppress HGP suggesting that naringenin suppresses glucose production in a PI3kinase independent manner. However, naringenin, similar to metformin, significantly decreased cellular ATP concentrations and increased p-AMP-activated protein kinase, which when activated, suppresses hepatic glucose output. To our knowledge, the effects of naringenin on HGP and similarity to the action of metformin are novel and intriguing and future studies using in vivo models are warranted.
This work is dedicated to my parents, my sister and my husband for always believing in me
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CHAPTER 1

1.1 INTRODUCTION

Type 2 diabetes mellitus is clinically defined by fasting or postprandial hyperglycemia or abnormally increased glucose excursion in response to a defined glucose load (1). Type 2 diabetes results in the abnormal metabolism of all three macronutrients, i.e., carbohydrates, proteins and lipids. Approximately 20.8 million Americans have type 2 diabetes and the incidence is increasing at an alarming rate. Globally, diabetes is expected to affect 366 million individuals by 2030. The health care costs in America alone were 132 billion dollars in 2001. Out of the total number of individuals with diabetes, 90-95% have type 2 diabetes. Type 2 diabetes is the leading cause of blindness, end stage renal disease and non-traumatic amputation. The etiological factors for type 2 diabetes include obesity, age, stress and family history. Obesity is the major risk factor for type 2 diabetes and 80% of people with diabetes are obese (2). Several cross sectional and prospective studies have shown a strong link between obesity and diabetes. The incidence of diabetes is approximately 2.9 times higher in over-weight subjects than in non-overweight subjects [(NAHANES III; (3)]. The association of obesity with diabetes is attributed to insulin resistance (4). Fluctuations in insulin sensitivity occur even during the normal life cycle for instance, decreased insulin sensitivity is observed during puberty, pregnancy and with ageing. In individuals with
normal β-cell function, glucose tolerance is preserved during conditions of insulin resistance as a decrease in insulin sensitivity is matched by an increase in insulin release demonstrating a non-linear relationship between insulin sensitivity and β-cell insulin response (5). In contrast, individuals at high risk for developing type 2 diabetes are unable to compensate for the decline in insulin sensitivity by a reciprocal increase in insulin secretion leading to impaired glucose tolerance and development of type 2 diabetes (5).

The increase in insulin resistance that accompanies obesity is a consequence of several factors including impaired inhibition of glucose output by insulin, increased insulin and free fatty acids in plasma and decreased glucose clearance by the skeletal muscle. In addition to the degree of fatness, the site of fat accumulation has an effect on insulin resistance. For instance, obese women with greater proportion of upper-body fat tend to be more insulin resistant, hyperglycemic and dyslipidemic than women with greater proportion of lower body fat (4; 5).

Nutrition is a major environment factor that has important implications in the development of obesity related disorders including type 2 diabetes. Therefore, dietary agents that have a potential to decrease body fat or improve hyperglycemia may be used as therapeutic agents for the treatment of diabetes and obesity. One such compound is the dietary lipid, conjugated linoleic acid (CLA). CLA is a potent weight loss agent that decreases adiposity in several species [reviewed in (6)] and therefore, may be effective in the attenuation of insulin resistance and type 2 diabetes.

Hepatic glucose production (HGP) is the main cause for increased fasting glucose levels in diabetes. In the second part of this project, I sought to develop and characterize
an in vitro model for testing the effects of bioactive compounds on HGP. Using this model, dietary CLA did not suppress HGP. However, I identified and characterized a novel effect of the citrus fruit flavonoid naringenin in suppressing HGP. I developed three aims to understand the mechanisms by which bioactive compounds, CLA and naringenin attenuate hyperglycemia, hepatic steatosis and insulin resistance.

1.2 AIMS

Aim 1: To determine the effectiveness and mechanism of action of dietary CLA in the attenuation of hepatic steatosis in a diet-induced model of obesity.

Dietary CLA attenuates hepatic steatosis and hyperglycemia in genetically obese rat models in conjunction with significant decreases in adipose mass. Therefore, it is not clear if the effects of CLA are due to an overall improvement in adiposity. We hypothesized that CLA will decrease hepatic steatosis in a diet induced model of obesity in rats by modulating genes involved in hepatic lipid metabolism.
Aim 2: To determine the role of adipokines in CLA mediated insulin resistance in mice.

Dietary CLA induces severe adipose tissue loss along with significant depletion of adiponectin and leptin in mice resulting in insulin resistance and hyperglycemia. Therefore, we hypothesized that maintenance of adipokines (by either removing CLA from the diet or using a TZD, rosiglitazone) attenuates insulin resistance in mice.

Aim 3: To determine the effectiveness and mechanism of action of the citrus fruit flavonoid naringenin in the attenuation of hepatic glucose production.

Naringenin is found in citrus fruits and has lipid lowering effects hypolipidemic activity in rodents. However, its role in the attenuation of hyperglycemia has not been investigated. Therefore, we hypothesized that naringenin will lower glucose production in Fao hepatoma cells by suppressing gluconeogenesis.
LITERATURE REVIEW

2.1 Pathophysiology of type 2 diabetes

Overt diabetes mellitus is a relatively late stage in the disease process. Significant defects in glucose and fuel homeostasis develop much before overt diabetes occurs (1). Insulin resistance is characterized by impaired glucose disposal as measured by a hyperinsulinemic-euglycemic clamp and is one of the earliest detectable metabolic derangements. Hyperinsulinemia is also detectable early in the disease process and is thought to be a compensatory mechanism for maintaining normal glucose levels. The adaptive response to insulin resistance involves changes in β-cell function and mass to maintain normal glucose tolerance in healthy individuals. However, the adaptive response is impaired in individuals who are at risk for developing type 2 diabetes, resulting in an inability to secrete sufficient insulin to maintain euglycemia. The hyperglycemic condition further worsens β-cell function through glucotoxic effects. In addition, elevated non-esterified fatty acids (NEFAs) associated with type 2 diabetes further contribute to β-cell dysfunction (1).

The characteristic elevation in blood glucose that accompanies type 2 diabetes may be attributed to 3 main factors. These include the following: (1) insulin resistance (IR) in which insulin mediated cellular events are blocked resulting in decreased
sensitivity to insulin in the liver, muscle and adipose tissue; (2) abnormal insulin secretion by the pancreas that interferes with glucose uptake by the peripheral tissues; and, (3) increased hepatic glucose production (HGP) which is the main cause for increased fasting blood glucose levels (FBG) associated with this condition (7). The liver is the main organ that contributes to fasting blood glucose via both gluconeogenesis and glycogen breakdown. Elevated free fatty acid levels (FFA/NEFA) in plasma are often associated with obesity, which is the main etiologic factor for type 2 diabetes (5; 7). Elevated plasma FFA induce insulin resistance in muscle, pancreas and liver. This induces hyperglycemia due to decreased clearance of glucose from blood, abnormal insulin secretion by the pancreas and impaired inhibition of gluconeogenesis by insulin. 

Figure 2.1 (p.7) illustrates the pathogenesis of type 2 diabetes (7).
Figure 2.1 Pathophysiology of type 2 diabetes (7)
2.2 Insulin resistance

The diminished ability of a given concentration of insulin to exert its usual biological effects is referred to as insulin resistance (8). Insulin resistance syndrome is a composite of abnormalities, including obesity, glucose intolerance, dyslipidemia and hypertension that eventually lead to type 2 diabetes (9). Liver, skeletal muscle and adipose tissue are the major sites for insulin resistance (7). Insulin resistance is characterized by increased glucose production by the liver, decreased glucose uptake and utilization by skeletal muscle, and increased lipolysis in the adipose tissue (7). Insulin resistance syndrome is increasing at an alarming rate and affects 27% of adults in the United States. Moreover, approximately 50% of severely obese children have the insulin resistance syndrome (9).

2.2.1 Hepatic glucose production

The liver plays a central role in both glucose and lipid metabolism. Hepatic insulin resistance is thought to be the main factor in the development of fasting hyperglycemia (9). Type 2 diabetes is associated with an increased rate of basal HGP despite 2- to 4- fold elevations in insulin concentrations suggesting hepatic resistance to insulin (7). In order to understand the contribution of liver insulin resistance to insulin resistance, liver insulin receptor knock out mice were generated (10). These mice developed hyperglycemia and compensatory hyperinsulinemia due to the inability to respond to insulin and suppress HGP. Increased HGP results from elevated glycogenolysis and gluconeogenesis. Hepatic gluconeogenesis alone contributes 50-60% of HGP and is thought to be primarily responsible for increases in fasting glucose levels in individuals with type 2 diabetes (7). Gluconeogenesis is mainly regulated by the
regulatory enzymes- phosphoenolpyruvate carboxy kinase (PEPCK), glucose 6-phosphatase (G-6-Pase), and fructose 1,6 bis-phosphatase. The rate of gluconeogenesis is regulated by hormones such as (1) insulin which suppresses HGP, (2) glucagon and glucocorticoids which stimulate gluconeogenesis, and (3) adipokines adiponectin and leptin which suppress HGP (7). Sustained hyperglycemia, a characteristic of type 2 diabetes, enhances gluconeogenesis by enhancing PEPCK and G-6-Pase gene expression (11). In addition to chronic hyperglycemia, high circulating concentrations of FFA stimulate HGP by increasing activities of PEPCK and G-6-Pase. Studies have shown that infusion of FFA in normal subjects and obese insulin resistant individuals enhances HGP by stimulating gluconeogenesis (12; 13) and is attributed to impairments in the insulin signaling pathway (14-17).

2.2.2 Muscle glucose uptake

Muscle glucose uptake accounts for the disposal of 70-90% of an oral glucose load. Insulin resistance in the muscle is one of the earliest defects during the progression to type 2 diabetes (9). This condition has been demonstrated by infusing insulin systemically or locally into the leg or forearm of individuals and measuring muscle glucose uptake (18). Classic studies by Randle et al. (19) proposed that FFA compete with glucose for oxidation in muscle which then contributes to impaired glucose oxidation and eventually to decreased uptake of glucose by the muscle. However, recent evidence using NMR spectroscopy showed that increases in FFA initially decrease glucose uptake by inhibiting transport, which then leads to decreased glucose oxidation and glycogen synthesis (20). Fatty acid mediated reduction in glucose transport is thought to be due to alterations in trafficking, budding, fusion or activity of GLUT-4 (21) as a
result of alterations in insulin signaling events (16). Taken together, these data suggest that glucose transport is the rate limiting step in insulin stimulated glucose uptake in the muscle. To clarify the role of muscle in insulin resistance, muscle specific insulin receptor (IR) knock out mice were generated (MIRKO). These mice have essentially normal blood glucose and insulin levels and are not insulin resistant. However, under hyperglycemic-euglycemic clamp conditions the muscles of MIRKO mice have 70-80% decreases in insulin stimulated glucose transport, glycolysis and glycogen synthesis. This demonstrates insulin resistance in the muscle despite having normal response to glucose tolerance testing. Interestingly, these mice have three fold increases in insulin stimulated glucose uptake in the adipose tissue compensating for the muscle insulin resistance [reviewed in (9)]. In contrast to the liver and muscle insulin resistance, adipose insulin resistance created by adipose tissue specific IR knockout mice have improved glucose and lipid homeostasis compared to normal mice [reviewed in (9)]. Although the mechanism for these intriguing findings remains unknown, it is proposed that insulin resistance in the fat cell changes the hormonal balance with increased levels of adiponectin and leptin and decreased tumor necrosis factor- (TNF) α expression.

### 2.2.3 Adipose tissue lipolysis

Lipolysis is defined as the hydrolysis of stored TG catalyzed by enzymes, releasing glycerol and FFA (22). The key enzymes that are involved in the hydrolysis of triglycerides (TG) in the adipose tissue include hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). These enzymes are regulated mainly by lipolytic and anti-lipolytic hormones, i.e., catecholamines and insulin, respectively (23). In addition, lipolytic enzymes are regulated by prostaglandins secreted from the adipose tissue
through paracrine signaling pathways. HSL is phosphorylated and activated by protein kinase A (PKA). These processes are mediated by the second messenger cAMP (24; 25). The catecholamines epinephrine and nor-epinephrine are secreted from the adrenal medulla and sympathetic nerves, respectively, and stimulate lipolysis by binding to G-protein coupled receptors known as β-adrenergic receptors and activating adenylate cyclase activity (26). Insulin inhibits lipolysis by activating phosphodiesterase 3B which degrades cAMP to AMP (25; 27). The resulting decrease in intracellular cAMP levels decreases PKA activity with concomitant reduction in HSL activity and lipolysis. Alterations in HSL gene have been linked with type 2 diabetes and knocking out HSL gene in mice is protective against diet induced insulin resistance coupled with decreased intra-muscular TG and circulating FFA (28). Because lipolysis from adipose tissue is suppressed by insulin in the fed state, insulin resistance is associated with increased FFA production from the adipose tissue further worsening the condition (29).

2.3. Insulin secretion

Pancreatic β-cells regulate insulin release in a very precise manner and can modify the quantity released according to the nature of stimulus, as well as the prevailing glucose concentration (5). Therefore, β-cells are crucial for maintenance of plasma glucose concentrations within a narrow range. Failure of β-cell is one of the key features in the development of type 2 diabetes (5). Glucose is the main nutrient secretagogue for the islet β-cell and glucose stimulated insulin secretion occurs by oxidative metabolism of glucose which results in an increase in the ATP/ADP ratio. Increases in cellular ATP concentration result in closure of the K⁺ ATP channels with depolarization of the plasma
membrane ultimately resulting in exocytosis of insulin from secretory granules (5). Glucose enters the β-cell through GLUT-2 via facilitated diffusion. The rate limiting step in glucose stimulated insulin secretion is glucokinase mediated conversion of glucose to glucose 6-phosphate. The glycolytic flux is dependent on the extracellular glucose concentration (30). Increased glucose metabolism results in accumulation of malonyl-CoA, an intermediate in the de novo fatty acid synthesis. Malonyl-CoA is an allosteric inhibitor of carnitine palmitoyl CoA (CPT-1), which regulates fatty acid transport into mitochondria for β-oxidation. Thus, the increase in malonyl-CoA results in accumulation of long-chain acyl-CoA and diacyl glycerol (DAG) that augment insulin release via activation of protein kinase C (PKC) (5). Although the most potent nutrient secretagogue for the islet β-cell is glucose, fatty acids also are important modulators of insulin secretion. Elevated plasma FFA released from the adipose tissue are thought to contribute to islet β-cell compensation that precedes the development of hyperglycemia in insulin resistant conditions (7). However, chronic elevation in FFA and glucose can cause β-cell failure by apoptosis, decreased sensitivity to glucose, and insulin synthesis resulting in type 2 diabetes (31-35).
2.4 Adipocytes as regulators of glucose homeostasis

Adipose tissue is traditionally viewed as an organ that primarily stores excess energy as fat. However, it is now recognized that alterations in adipose tissue have profound implications for glucose homeostasis as either too much fat (obesity) or too little fat (lipodystrophy) can both result in insulin resistance and hyperglycemia. Furthermore, it is now well established that the adipose tissue is involved in immune response, regulation of blood pressure, and thyroid and reproductive function, mainly by the synthesis and release of peptide hormones [reviewed in (29)].

2.4.1 Leptin

Leptin was the first adipokine discovered that has a role in modulating adiposity by repressing food intake. Mutation in the leptin gene results in obesity in both humans and animals (36). In addition to its role in energy balance, leptin plays an important function in the maintenance of glucose homeostasis. Leptin has been shown to decrease hyperglycemia in leptin deficient ob/ob mice, lipodystrophic mice and humans before body weight is corrected (37; 38). The anti-obesity and anti-hyperglycemic effects of leptin are not solely due to its anorectic effects. In addition, leptin has been shown to improve insulin sensitivity in the muscle through direct activation of the AMP-activated protein kinase (AMPK) pathway (39).

2.4.2 Adiponectin

Adiponectin (AdipoQ or Acrp 30) is a 30- kDa protein with an amino terminal collagen domain and a carboxy terminal globular domain and can exist as a trimer, hexamer or multimer with 12-18 subunits. Two types of receptors have been identified-adipoR1 and adippoR2. They contain two similar trans-membrane proteins with similar
homology to G-protein-coupled receptors (40; 41). A second molecule, T-cadherin, lacks a transmembrane domain and acts as a co-receptor for the high-molecular form on endothelial and smooth muscle cells (42). Adiponectin is a relatively abundant plasma protein (5-10 µg/ml) accounting for approximately 0.01% of all plasma proteins (43; 44). Plasma levels of adiponectin are inversely associated with body mass and type 2 diabetes [reviewed in (29)]. Plasma adiponectin levels are markedly reduced in people with type 2 diabetes and the magnitude of adiponectin reduction is strongly correlated with the severity of insulin resistance in peripheral tissues and liver [reviewed in (7)]. Adiponectin increases insulin sensitivity by inhibition of hepatic glucose production along with increased peripheral glucose clearance (45). These effects appear to involve activation of AMPK in the liver and skeletal muscle. The beneficial effects of adiponectin on insulin sensitivity are further emphasized by the marked increase in adiponectin levels by treatment with the insulin sensitizing agents known as thiazolidinediones (TZD) (46-49). Adiponectin exists in the serum in two forms, as a low molecular weight (LMW) complex composed of hexamers and a high molecular weight complex (HMW) of 12-18 subunits (50). Furthermore, increases in the ratio of HMW/total adiponectin rather than total adiponectin correlate with improved insulin sensitivity. TZD treatment has been shown to significantly increase the levels of HMW adiponectin in db/db, ob/ob mice and in humans (46). In addition to its anti-diabetic effects, adiponectin has been shown to exert anti-inflammatory and anti-atherosclerotic effects in animals and humans (7).

### 2.4.3 Tumor necrosis factor (TNF-α) and other cytokines

There is increasing evidence that suggests that both obesity and type 2 diabetes are inflammatory states. One such adipose derived inflammatory cytokine is TNF-α, that
plays an important role in insulin resistance. Elevated TNF-α levels have been reported in obesity and other insulin resistant states (51). In addition to TNF-α, the cytokine interleukin (IL-6) is secreted by adipocytes and is implicated in muscle insulin resistance and β-cell apoptosis (7; 52). When TNF-α is infused into rodents, it is associated with development of insulin resistance, increased lipolysis, activation of inflammatory mitogen activated protein kinase (MAPK) isoforms including c-Jun N-terminal kinase 1 (JNK1) that mediates serine phosphorylation of insulin receptor substrate 1 (IRS-1), activation of nuclear factor κB (NF-κB), induction of suppressor of cytokine signaling 3 (SOCS3), and production of ROS [reviewed in (29)].

2.4.4 Resistin

Resistin (FIZZ3) is an inflammatory molecule secreted by the adipose tissue that has hyperglycemic activity. Levels of resistin are elevated in human type 2 diabetes and obesity, and correlate mainly with increased hepatic glucose output. When infused into normal rodents, resistin causes severe hepatic insulin resistance, but has no effect on muscle insulin sensitivity. However, there is considerable controversy surrounding the role and source of resistin in humans [reviewed in (7; 29)].

2.4.5 Non-esterified fatty acids (NEFAs)/free fatty acids (FFA)

NEFAs are released from the adipose tissue during fasting and serve as an energy source. Under conditions of fasting, NEFAs decrease adipocyte and muscle glucose uptake and increase hepatic glucose production and thus, have a glucose sparing effect for neurons and erythrocytes requiring glucose for energy. During the fed state, insulin inhibits NEFA production by suppressing adipose tissue lipolysis. In contrast, during insulin resistant states, chronic elevation in NEFA levels causes a decrease in insulin
secretion by inducing lipotoxicity and apoptosis of islet cells. In addition, elevated NEFAs inhibit the ability of insulin to promote peripheral glucose uptake and reduce hepatic glucose production, promoting insulin resistance and hyperglycemia (7; 29).

2.5 Ectopic fat storage and insulin resistance

Obesity is often associated with a positive energy balance and the excess lipid is stored as triglyceride in the liver and skeletal muscle [reviewed in (4)], resulting in insulin resistance and type 2 diabetes. Weight loss can substantially decrease the ectopic fat accumulation attenuating insulin resistance. Thiazolidinediones, widely used for the management of type 2 diabetes, are peroxisome proliferator activated receptor-\(\gamma\) (PPAR-\(\gamma\)) agonists that preferentially increase subcutaneous but not visceral fat and possibly decrease ectopic fat deposition [reviewed in (4)]. In addition to obesity, ectopic fat deposition occurs in lipodystrophy that is characterized by almost complete ablation of adipose tissue mass but increase in visceral fat mass, resulting in insulin resistance and diabetes. Animal studies show that when adipose mass is ablated by blocking adipose tissue development, mice develop hepatic and muscle steatosis (53; 54) and these effects can be reversed by transplantation of adipose tissue back into these animals along with reversal of insulin resistance and diabetes (55).

2.5.1 Mechanisms for ectopic fat storage

i. Abnormal fat cell

Evidence from studies with Pima Indians demonstrates that impairment in proliferation and differentiation capacity of adipocytes is a predisposing factor for the development of type 2 diabetes. In addition, enlarged fat cells in the abdominal region
predict the development of diabetes independently of insulin resistance and insulin secretion. Data from several studies suggest that enlarged fat cells reflect a failure of adipocytes to differentiate/or proliferate, processes that are regulated by complex paracrine, endocrine and metabolic signals [reviewed in (4)]. The regulation of adipocyte differentiation and proliferation involves coordinate activation/inactivation of nuclear transcription factors, e.g., PPAR-γ, ADD/SREB-1, CCAAT/enhancer-binding proteins (C/EBP)-α, β and δ (56). These transcription factors regulate downstream genes required for lipid storage and insulin sensitivity and are regulated by external factors such as prostaglandins, cytokines, and hormones including insulin. Defects in any one of these steps can result in failure to proliferate and differentiate [reviewed in (56)].

**ii. Impaired fat oxidation**

Another explanation for increased intracellular lipid is decreased whole body fat oxidation. Inhibition of fat oxidation in rodents increases intracellular lipids and decreases insulin sensitivity. Increased respiratory quotient (RQ) is indicative of decreased post absorptive fat oxidation and weight gain in humans. Studies in humans show a large degree of inter-individual variability in the ability to oxidize fat and that a low capacity to oxidize fat is associated with higher fasting insulin levels [reviewed in (4)]. Several factors modulate fat oxidation by the skeletal muscle. For instance, chronic exercise increases muscle uptake of lipid and increases in rate limiting enzyme activities such as CPT-1 and the number of mitochondria. Endocrine and neural-endocrine systems also regulate ability to oxidize fat, e.g., adiponectin and leptin secreted by the adipose tissue have been shown to increase fat oxidation. Any defects in regulators of oxidation can impair the ability to oxidize fat and lead to ectopic fat accumulation (4).
2.5.2 Example of ectopic fat storage: Non alcoholic fatty liver disease

Non alcoholic fatty liver disease (NAFLD) is one of the most common liver disorders and is an aggregate of disorders related to obesity, insulin resistance, type 2 diabetes, hypertension and hyperlipedemia (57-59). It is a progressive disorder that includes a spectrum of liver diseases ranging from simple steatosis to fibrosis, non-alcoholic steatohepatitis (NASH), cirrhosis and end-stage liver disease (60; 61). Epidemiological studies have reported that approximately 20-30% of the adults in the United States and other western countries have NAFLD (62). Obesity is very closely associated with NAFLD and data from the Third National Health and Nutrition Examination Survey (NHANES III) reported that 9.1 million of the 47 million persons in the United States who have insulin resistance, also have NAFLD (63). The pathogenesis of NASH although not fully understood, is explained by the “two hit hypothesis” proposed by Day et al. (64). The first hit occurs with the accumulation of fat in the liver. The progression from simple steatosis to NASH occurs in the presence of a second hit which usually involves inflammation, oxidative stress, and mitochondrial injury (64).

i. Fatty acids and NASH

Lipids accumulate in hepatocytes when input (uptake of lipid or synthesis) exceeds the output (oxidation or export) (58; 65). Although triglycerides (TG) are the predominant lipids that accumulate in the liver, it is not uncommon to find accumulations of cholesterol, diacyl glycerol (DAG), free fatty acids and phospholipids (65). The regulation of hepatic lipid balance involves cooperation between the liver and adipose tissue. In addition, hormones such as insulin, glucocorticoids, glucagon and nor-epinephrine regulate the balance between lipid mobilization to and from adipose tissue.
and the liver (65). For instance, during fasting, lipids are hydrolyzed in the adipose tissue, increasing plasma FFA that are taken up by the liver and either re-esterified into TG and secreted as VLDL or oxidized in the mitochondria, peroxisomes or microsomes. Studies using mitochondrial β-oxidation inhibitors and more recently, peroxisomal oxidation disruption have been shown to promote fatty liver (66). The increased fat in the liver promoted liver injury when other insults such as alcohol or hepatitis are superimposed. FFA promote hepatocyte toxicity either directly by membrane disruption and inhibition of Na⁺/K⁺ ATPase or indirectly via increased lipid peroxidation and increases in reactive oxygen species (ROS) (58). One mechanism by which fatty acids act as cytotoxic agents in hepatocytes is by up-regulating the expression of pro-apoptotic molecule Fas (CD95) in hepatocytes. The increased expression of Fas has been reported in livers of patients with NASH (67). In addition to fat, studies in mice have shown that a high carbohydrate diet can also up-regulate Fas expression (68). Increased Fas in turn sensitizes the cells to cell death from attack by inflammatory cells e.g. cytotoxic T cells or natural killer cells that express the Fas ligand (68). Fatty acids may induce apoptosis of non-liver cells by destabilizing lysosomes and releasing proteases such as cathespin B into the cytoplasm. In addition to acting as a protease, cathespin B activates NF-κB, increasing hepatocyte expression of cytokines IL-1 and IL-6, suppressors of cytokine signaling (SOCS), and TNF-α resulting in hepatocyte injury and inflammation. Blocking TNF-α improves hepatic steatosis suggesting that induction of this inflammatory molecule can induce both apoptosis and steatosis (69).
ii. Oxidative stress and NASH

Fat-laden hepatocytes have been shown to be prone to mitochondrial dysfunction (70). While steatosis is not associated with abnormalities in the capacity for fatty acid uptake into mitochondria or β-oxidation, it impairs the function of the mitochondrial respiratory chain complex. The respiratory chain complex recycles NADH and FADH$_2$ to NAD and FADH along with conversion of ADP to ATP (71). Therefore, while the electron transport is impaired in fatty livers, fatty acid oxidation occurs at normal or even increased rates. The elevated fatty acid β-oxidation places an additional stress on the mitochondrial respiratory chain increasing the production of ROS. Increased levels of ROS increase lipid peroxidation, which is detrimental to mitochondrial DNA (58; 59; 72; 73). While the initial trigger for fatty acid induced respiratory chain dysfunction is unknown, increased TNF-α levels have been postulated to initiate the damage. Recently, a study involving 18 patients with NASH demonstrated diminished activity of all 5 hepatic mitochondrial respiratory complexes (I, II, III, IV and ATP synthase) (71). Hepatic steatosis triggers hepatic insulin resistance by alterations in the insulin signaling pathway discussed in detail in section 2.6.3.

2.6 Insulin signaling

2.6.1 Signal Transduction

Insulin signaling is mediated by a complex system consisting of highly integrated networks. The signaling cascade is triggered when insulin binds to its receptor (IR). IR belongs to a subfamily of receptor tyrosine kinases along with insulin like growth factor-1 receptor and insulin receptor –related receptor (74). The IR is a heterodimeric protein
complex consisting of two extracellular \( \alpha \)-subunits that bind insulin and two intracellular \( \beta \)-subunits. Two of the \( \alpha-\beta \) dimers are linked by disulfide bonds to form a tetramer. The \( \alpha \)-subunit acts like an allosteric factor and inhibits the tyrosine kinase activity of the \( \beta \)-subunit. Binding of insulin to the \( \alpha \)-subunit or removal by knocking out the \( \alpha \)-subunit removes the inhibition on the \( \beta \)-subunit, thereby initiating the activation process. The subunits then undergo a trans-phosphorylation reaction defined as the transfer of a phosphate group by a kinase to a residue within the same kinase or to a different kinase molecule, further increasing their kinase activity. Binding of insulin to the IR results in phosphorylation of insulin receptor substrates (IRS). More than ten substrates of the IR have been identified so far and they vary in their tissue distribution and sub-cellular localization. IRS proteins have a pleckstrin homology (PH) domain, a protein tyrosine binding domain (PTB) and several tyrosine residues that can undergo phosphorylation by the IR tyrosine kinase. The IRS proteins are linked to the activation of two main pathways, viz., the phosphatidylinositol 3-kinase (PI3K)-Akt/protein kinase B (PKB) pathway and the ras-mitogen-activated protein kinase pathway (MAPK). The PI3K pathway is responsible for most of the metabolic effects of insulin. After phosphorylation of the tyrosine residues by the IR, the IRS proteins bind to intracellular molecules containing Src-homology-2 domains (SH2 domains) which in turn become activated and associate with other downstream molecules leading to a complex cascade of events. One such key intermediate is phosphatidylinositol 3-kinase (PI3-kinase) which exits as a heterodimer. The molecule consists of a p110 catalytic subunit and a p85 \( \alpha \) regulatory subunit. Inhibition of PI3kinase blocks the effects of insulin on glycogen synthesis, lipid synthesis, glucose uptake and adipocyte differentiation. Activated PI3kinase produces
phosphoinositides- 3,4,5-triphosphate (PIP₃) from PIP₂ at the plasma membrane, which activate two kinases, PDK1 and PDK2. PDK1 phosphorylates Akt (protein kinase B) at threonine-308 while PDK2 phosphorylates Akt at serine-473. When Akt/PKB is phosphorylated, it is also activated to regulate several downstream enzymes involved in glycogen synthesis, gluconeogenesis, glycogenolysis, lipolysis and apoptosis. Akt is a serine/threonine kinase that mediates most of the metabolic actions of insulin through PI3Kinase. Activation of Akt leads to phosphorylation of several substrates including other kinases and transcription factors. There are three different isoforms of Akt in mammals encoded by different genes and involved in different biological functions. For instance, deletion of Akt1 results in growth retardation and deletion of Akt2 results in development of insulin resistance and diabetes due to inability of insulin to stimulate glucose transport and inhibit glucose output. Akt3 deficient mice do not develop diabetes but have neural defects. Akt2 is mainly distributed in insulin sensitive tissues such as liver, muscle and adipose [reviewed in (9; 74)]. **Figure 2.2 (p.25)** summarizes the insulin signaling pathway and metabolic effects of insulin signaling.

### 2.6.2 Akt targets

**i. Glycogen synthase kinase-3 (GSK3):** GSK3 was the first physiological target of Akt to be identified. Phosphorylation of GSK3 by Akt decreases its activity which releases its inhibition on glycogen synthase resulting mainly in increased glycogen synthesis (75).
ii. **Rab-GTPase-activating protein, AS160**: Akt increases glucose translocation by phosphorylating and inhibiting the Rab-GTPase-activating protein, which in turn activates Rab small GTPases required for GLUT 4 translocation to the plasma membrane by re-organization of the cytoskeleton (76).

iii. **Mammalian target of rapamycin (mTOR)**: mTOR plays an important role in the regulation of protein synthesis and growth. mTOR activity is inhibited by tuberin-hamartin complex. Insulin mediated phosphorylation of this complex releases its inhibition of mTOR pathway (77).

iv. **Forkhead family of transcription factors (FOX)**: FOX family of transcription factors contains over 100 members that are involved in the regulation of glucose and lipid metabolism. For instance, FOXO1 activates hepatic gluconeogenic genes and inhibits adipogenesis. FOXO2 is a regulator of fasting lipid metabolism. Phosphorylation of FOXO1 and 2 by Akt inhibit their transcriptional activity (78).

v. **Sterol regulator element binding protein (SREBP-1c)**: While many of the metabolic effects of insulin on glucose metabolism are mediated by FOXO1, its effects on lipid metabolism are mediated by a member of the SREBP nuclear hormone receptor family of proteins, SREBP-1c. SREBP-1c is the dominant form in the liver and adipose tissue and regulates genes involved in lipid synthesis such as fatty acid synthase (FAS) and stearoyl CoA desaturase (SCD-1). SREBP 1c is also involved in the regulation of gluconeogenic enzymes such as PEPCK, although to a lesser extent than FOXO-1. SREBP precursor proteins are membrane bound and mainly associated with the endoplasmic reticulum by Insig proteins. Activation of SREBP by insulin or depletion of sterols results in dissociation from the Insig proteins and translocation to the nucleus to
activate transcription. Insulin increases SREBP-1c transcription, maturation and activity. Insulin increases SREBP transcription by activating liver X receptor (LXR), a transcription factor that binds to and activates SREBP-1 transcription. Additionally, insulin inhibits Insig expression. Insulin is also thought to phosphorylate SREBP-1c mainly by activation of PKC λ (9).
Figure 2.2 Insulin signaling pathway (9)
2.6.3 Molecular mechanisms of insulin resistance

Insulin resistance can be attributed to multiple mechanisms such as decreased synthesis, increased degradation of IR and signaling molecules, inhibitory serine phosphorylation of IRS proteins, alteration in the ratios of signaling molecules, and interaction of IR with inhibitory proteins (Figure 2.3, p.29). For instance, recent studies have demonstrated that an increase in SREBP-1c can result in a decrease in IRS-2 transcription (79). Insulin receptor and IRS proteins can undergo phosphorylation at the serine residue by protein kinase C (PKC), extracellular signal regulated kinase (ERK), JNK and IKKβ which decreases its activity. Insulin resistance can also be produced by interaction of inhibitory proteins with molecules in the insulin signaling pathway. For example, inflammatory cytokines induce suppressors of cytokine signaling proteins (SOCS) which bind to the IR and block its signaling (9; 80-82).

The molecular mechanisms of insulin resistance have been demonstrated extensively by Shulman et al. in rodents and humans over the past two decades. Using $^{13}$C magnetic resonance spectroscopy (MRS) to measure intracellular levels of glycogen synthesis in the muscle of individuals with type 2 diabetes, Shulman et al. (20) demonstrated that insulin stimulated muscle glycogen synthesis was decreased by over 50% in patients with type 2 diabetes compared to normal individuals under hyperinsulinemic (~80μU/ml)-hyperglycemic (10mmol/l) clamp conditions. Furthermore, using $^{13}$C and $^{31}$P MRS studies to measure intracellular levels of glucose, glucose-6-phosphate and glycogen, Cline et al. demonstrated that glucose transport was the rate limiting step in insulin stimulated glycogen synthesis in muscle (83). In addition, studies with offspring of subjects with type 2 diabetes exhibited similar abnormalities (84; 85).
Results demonstrated that fasting plasma fatty acids were a good predictor for insulin resistance in the healthy cohort with type 2 diabetic parents. Subsequent studies using $^1$H MRS to detect intramyocellular lipid showed that lipid content in the muscle was a better predictor for insulin resistance than plasma fatty acids in both adults and children (86).

The mechanisms by which FFA induce insulin resistance in both humans and rodents have been elucidated (16; 87-89). In the skeletal muscle, accumulation of intramyocellular fatty acyl CoAs and DAG from plasma or impaired $\beta$-oxidation activate serine/threonine kinases such as PKC (PKC-θ in rodents and PKC-δ in humans). Activated PKC-θ/δ phosphorylates IRS-1 on the serine residue instead of tyrosine residue which seems to negatively regulate IRS signaling. Similar to FFA, cytokines also induce similar defects in insulin signaling observed in insulin resistant states. Phosphorylation of serine residues in IRS-1 decreases insulin stimulated Akt2 activity, thereby decreasing GLUT-4 translocation and glucose uptake by the skeletal muscle [reviewed in (74)].

In the liver, increased levels of DAG resulting from elevations in plasma FFA or suppressed $\beta$-oxidation activate PKC-ε leading to reduced IRS-2 tyrosine phosphorylation (87). This decreases activation of glycogen synthase and glycogen synthesis, and increases glucose output due to impaired FOXO inhibition. In addition to these data, when certain Ser/Thr kinases (e.g., JNK, IKK-β, PKC-θ) were either knocked down or inhibited using pharmacological inhibitors, high fat diet induced insulin resistance was prevented in rodent models [reviewed in (90)]. Furthermore, recent studies in mice with specific Ser to Ala mutations in muscle IRS-1 (IRS-1 Ser-Ala302, Ser-Ala307 and Ser-Ala612) revealed protection against high-fat diet-induced resistance (90). Similarly, when mitochondrial glycerol-3phosphate acyl transferase, a key enzyme in de
novo lipogenesis is knocked down, high fat induced synthesis of DAG is inhibited resulting in decreased PKC-θ activation and fat-induced liver insulin resistance (91). Collectively, these data suggest that phosphorylation of key Ser residues on IRS plays an important role in the development of muscle and liver insulin resistance and have important implications for the development of novel treatment agents to prevent insulin resistance and NAFLD.
Figure 2.3 Molecular mechanisms of insulin resistance (9)
2.6.4 Mitochondrial dysfunction in insulin resistance

Mitochondrial dysfunction, whether associated with β-cell dysfunction or impaired oxidative and phosphorylative capacity of the skeletal muscle, plays an important role in the pathogenesis of type 2 diabetes. Petersen et al. (92) demonstrated a 40% decrease in muscle oxidative and phosphorylation capacity along with increased intramyocellular and intra-hepatic lipid content in healthy lean elderly subjects with severe insulin resistance compared to body mass index (BMI) and activity matched young control subjects. These data suggest that ageing may predispose individuals to loss of mitochondrial function which results in insulin resistance. Similar results in loss of mitochondrial function was observed in young lean insulin resistant offspring of parents with type 2 diabetes (93). In addition to an 80% increase in intramyocellular lipid content in these subjects, mitochondrial density was decreased by 38% determined by electron microscopy suggesting that mitochondrial dysfunction may be a predisposing factor for the development of insulin resistance. Key factors that have been identified as regulators of mitochondrial density in the skeletal muscle include peroxisome proliferator-activated receptor-γ co-activator (PGC)-1α and AMPK. The cellular switch, AMPK, is activated during exercise and ischemia when there is a decrease in the ATP/AMP ratio. AMPK mediates its effects on increasing mitochondrial biogenesis via increase in PGC-1α [reviewed in (90)]. It thus seems that the phenotype of insulin resistance depends on the components and tissues in which insulin signaling is altered and therapy should be directed at the specific pathway and tissue affected.
2.7. Bioactive compounds

Bioactive compounds are non-essential dietary compounds that occur in small amounts in foods. Many bioactive compounds have been a subject of intense research for the treatment/prevention of several chronic diseases such as cancer, cardiovascular diseases and diabetes. Before dietary recommendations can be made, much scientific research needs to be conducted to understand mechanisms, safety and efficacy of these compounds. Here, we investigated the effects of two bioactive compounds, viz., 1) naturally occurring dietary fatty acid, conjugated linoleic acid (CLA) and, 2) citrus fruit flavonoid naringenin, on type 2 diabetes.

2.7.1 Conjugated linoleic acid

i. Structure and synthesis

The dietary lipid conjugated linoleic acid (CLA) is a group of fatty acids which consists of several conjugated and stereoisomeric alterations of linoleic acid (Figure 2.4, p.33) (6; 94; 95). Although the presence of this novel fatty acid in milk fat was discovered in the early 1930s, the biological effects of CLA were not recognized until much later. Kramer et al. (96) determined the cis9trans11 (c9t11) as the principal CLA isomer in dairy products in 1977. Natural forms of CLA are mainly found in ruminant sources such and meat and dairy products. Milk fat is the richest source of natural CLA and typically contains 4-6mg/g lipid. Dietary variations influence the amount of this lipid milk (95; 97). Natural forms of CLA exist primarily as the c9t11CLA isomer (80-90%). CLA is also known as rumenic acid as it is produced by microbial conversion in the rumen and by delta 9 desaturation of vaccenic acid in the mammary gland [reviewed in (94; 97)]. Of the 16 different isomers of CLA currently identified, the c9t11-CLA found
naturally, and the $trans10cis12$ ($t10c12$) found mainly in synthetic products are the most studied. Other isomers of CLA consist of cis/trans, trans/cis isomers and ~2-5% exist as cis/cis and trans/trans isomers (97). Measurements of human adipose tissue have shown the presence of c9t11-CLA to be highly correlated with milk fat consumption (98). The average intake of CLA in an omnivore diet is approximately 150mg/day for women and 200mg/day for men (99). CLA predominantly the c9t11 isomer can be produced by the process of bio-hydrogenation of $\alpha$-linoleic acid with anaerobic ruminant bacteria, such as *Butyrivibrio fibrisolvens*. In addition to natural synthetic process, CLA can now be produced chemically using several methods [reviewed in (94; 97)]. Most of the CLA that is currently being used in feeding studies is synthesized by alkaline isomerization of linoleic acid found mainly in vegetable oils. CLA produced in this manner is mostly available in a 1:1 ratio of c9t11- and the t10c12-CLA isomers (6).
Figure 2.4 Structure of CLA and LA (97), Chapter 2
**ii. Biological activity**

*Cancer:* The first biological activity of CLA was observed by Pariza and colleagues who reported that grilled ground beef had mutagenic inhibitory activity and attributed this property to CLA (100). Subsequent studies by Ip *et al.* (101; 102) showed that CLA decreased carcinogen induced mammary carcinogenesis in rats. Furthermore, the lowest level of CLA that inhibited tumor number in their rat model was 0.1% (w/w). Compared to fish oil, which is effective at doses exceeding 10% in the diet, CLA was found to have 100-fold greater efficacy (97). CLA supplementation inhibits carcinogenesis in several animal models. For instance, administration of CLA inhibits dimethylbenz(a)anthracene-induced tumorigenesis of the mammary (101), skin (103) and forestomach (104) when supplemented at 0.5-1.5g/100g diet [reviewed in (97)]. CLA also inhibits growth of transplanted tumor cell lines derived from mammary and prostate cancers (105). However, CLA does not inhibit carcinogenesis in all animal models. For instance, CLA had no effect on the growth of transplanted prostate and breast cancer cells in the Apc\textsuperscript{Min} mouse model (106). Several mechanisms have been proposed for the anti-cancer effects of CLA and include reduction of cell proliferation, alteration of the cell cycle, induction of apoptosis, altered eicosanoid formation, and changes in gene expression [reviewed in (107)]. Apart from studies in experimental models of carcinogenesis, there are few studies showing anti-tumor effects of CLA in humans (107). However, because CLA exerts its anti-cancer effects in various animal models of carcinogenesis, it may have potential beneficial effects in human carcinogenesis and merits further research.
**Diabetes:** Of all the biological effects of CLA, its role in diabetes is the most controversial. The first study was published in 1998 by Houseknect *et al.* (108) who reported the beneficial effects on feeding 1.5% mixed isomer (MI)-CLA for 14 days in Zucker diabetic fatty rats (ZDF). Feeding a diet with CLA improved hyperinsulinaemia and normalized glucose tolerance in rats. In a subsequent study, Ryder *et al.* (109) reported similar improvements in glucose tolerance and insulin action in ZDF rats fed a 50:50 MI-CLA compared to rats fed a control diet or diet supplemented with only c9t11-CLA isomer. This suggested that the hypoglycemic effect was mainly attributed to the t10c12-CLA isomer. They further showed that the 50:50 MI-CLA improved insulin-stimulated glucose uptake and glycogen synthase activity in skeletal muscle along with significant decreases in body weight after 14 days of CLA supplementation. However, there was little change in glucose uptake in ZDF rats fed the c9t11-CLA diet. Henriksen *et al.* (110) gavaged obese Zucker (fa/fa) rats with either a control diet (corn oil) or 1.5% CLA for 21 days. To determine the isomer-specific effects, rats were fed either a 50:50 MI-CLA or individual c9t11- or t10c12-CLA isomers. Both MI-CLA and the t10c12-CLA isomer alone significantly improved glucose tolerance and decreased plasma insulin, free fatty acids and glucose compared with the control diet and the c9t11-CLA containing diet, supporting the hypothesis that the anti-diabetic effects of CLA in rats is due to the t10c12-CLA isomer. Nagao *et al.* (111) recently showed that 1% MI-CLA significantly increased serum adiponectin levels in ZDF rats fed CLA for 8 weeks. In addition, several studies in rats show that CLA decreases hepatic TG levels by increasing the expression of lipid oxidative genes (112-114).
In contrast to these findings in rats, several in vivo studies in mice show detrimental effects of feeding dietary CLA. Chronic supplementation with either 0.5% or 1% CLA mixture or purified t10c12-CLA has been shown to cause rapid loss of adipose tissue in association with severe hepatic steatosis and insulin resistance (115-120). In the liver, CLA increases adipogenic genes, e.g., PPAR-γ, fatty acid transporter (FAT/CD36), adipocyte lipid binding protein (ALBP/ap2) which are involved in fatty acid uptake and trafficking (117). PPAR-γ overexpression in the livers of A-ZIP/F1 transgenic mice produces a similar phenotype (121). Furthermore, CLA increases the expression of lipogenic genes such as ATP citrate lyase, malic enzyme, fatty acid synthase (FAS), acetyl CoA carboxylase (ACC) and SCD-1 in mice (117; 122). In addition to liver steatosis, CLA feeding has been shown to induce insulin resistance, hyperglycemia and hyperinsulinaemia in mice (117; 118). The increase in insulin levels has been recently attributed to β-cell hyperplasia and is speculated to be secondary to suppression of PPAR-γ gene by CLA (117; 118).

The chronology of CLA-mediated lipoatrophic syndrome is shown in Figure 2.5 (p.38) (119). CLA alters gene expression in murine adipose within two days and the adipose tissue seems to be the first target. Feeding CLA initially increases the production of inflammatory cytokines such as TNF-α and IL-6/8 causing rapid depletion of adipose stores by apoptosis and suppression of lipogenic genes, mainly PPAR-γ. The decrease in fat stores is associated with significant reduction in plasma levels of the insulin sensitizing adipokines – adiponectin and leptin (118; 120). Tsuboyama-Kasaoka et al. (116) demonstrated that the hepatic steatosis and hyperinsulinaemia can be attenuated by leptin infusion to CLA-fed mice. Insulin resistance induced by CLA eventually leads to
compensatory β-cell hyperplasia and increased plasma insulin levels. The chronic hyperinsulinaemia is thought to lead to the development of hepatic steatosis (119).

Human studies have generated mixed effects of CLA on type 2 diabetes. Noone et al. (123) showed that a supplement combining the two main CLA isomers significantly decreased plasma triacylglycerol and very low density lipoprotein (VLDL) levels compared to linoleic acid in healthy human subjects. In contrast, randomized, placebo controlled studies in obese men with metabolic syndrome and in patients with type 2 diabetes supplemented with either 3g/d mixed CLA isomers(124) or individual isomers (3.4g/d t10c12(124) and 3g/d purified c9t11 isomer (125)) showed adverse effects on insulin sensitivity and glucose metabolism. Interventions in most human trials have lasted from 8-12 weeks. Wargent et al. (126) showed that prolonged treatment with CLA or CLA enriched with t10c12-CLA improved glucose tolerance and decreased plasma insulin levels in female ob/ob mice. CLA supplementation was associated with initial increases in fasting glucose, insulin levels along with worsened glucose tolerance. However, CLA had beneficial effects after 10 weeks of supplementation. Therefore, is possible that in humans, the detrimental effects of CLA on insulin sensitivity are transient, and supplementation with CLA for longer duration may help to clarify the role of dietary CLA in the attenuation of diabetes.
Figure 2.5 Chronology of lipoatrophic syndrome induced by t10,c12-CLA in the mouse (119)

1) decrease in fat stores; 2) adipokine-mediated insulin resistance; 3) beta-cell proliferation; 4) hyperinsulinemia; 5) liver steatosis.
**Obesity:** CLA is currently marketed as a weight loss supplement and it is claimed to decrease body fat and increase lean body mass. The anti-obesity effects of CLA were first demonstrated by Park et al. (127) in ICR mice fed 0.5% mixed isomers of CLA. CLA supplementation for 4-5 weeks resulted in a 60% decrease in body fat mass and 5-14% increases in lean body mass compared to control mice fed corn oil. The body fat lowering effect of CLA has been demonstrated in several species including mice, rats, chickens, pigs and humans [reviewed in (6; 97)]. Compared to other species, mice seem to be most responsive to the lipid lowering effect of CLA. The average reductions in body fat in mice fed CLA have been approximately 50% and reductions of up to 70% have been reported. Adipose lowering effects of CLA have also been reported in Sprague Dawley, ZDF and Otsuka Long Evans Tokushima Fatty (OLETF) rats, although the effects were not as dramatic as in mice (6; 97). Similar effects on decreasing body fat and increasing lean mass have been observed in pigs (128). Studies using pair-fed animals have shown that the lipid lowering effects of CLA occur independently of food intake (109; 129). It is now recognized that the t10c12-CLA isomer is mainly responsible for lowering body fat in mouse and rat models (109; 112; 115; 119).

The effect of CLA on body composition changes in humans is not clear. Studies using either mixed isomers of CLA or c9t11- or t10c12-CLA had moderate or no effect on body fat reduction [reviewed in (6)]. Malpuech-Brugere et al. (130) examined the isomer specific effects of CLA on changes in body composition in overweight middle aged adults (n=81) using 1.5 or 3g c9t11 and t10c12 isomers in a double blind, randomized, placebo controlled trial. CLA was supplemented as a triacylglycerol in a drinkable dairy product for 18 weeks. Body composition changes were measured using
DEXA at the end of the trial. There was no significant difference in body composition of treated and control groups. Krieder et al. (131) evaluated the effects of CLA supplementation on body composition in 24 resistance trained males. Subjects were randomized into CLA or control groups and received either 6g CLA or placebo for 28 days. Body composition changes were measured by DEXA at the end of the study and no significant differences were reported in this short term study although trends for improved strength and performance were reported for the CLA group.

In contrast to these studies, Blankson et al. (132) tested the dose response relationship between CLA and body fat mass in obese human subjects and showed that CLA supplementation decreased body fat mass when CLA was provided as a mixed isomer over a period of 12 weeks. Body composition changes were monitored over time and significant differences were observed at doses above 3.4g CLA/day. Other studies using CLA show increases in lean body mass (LBM) with exercise training (97; 133). It is however, important to note that the extent of body fat mass loss in humans is 40-50% lower than in mice (6). These differences may be due to the duration, dose of CLA administered, and age of species. In general, studies in mice are conducted with much higher dose (approximately 5 times more CLA per kg body weight) than humans. Furthermore, almost all studies have been conducted with growing animals. Future studies in humans with higher does and longer duration may help to clarify the role of CLA in humans (134).
iii. Mechanism of action of CLA

**Lipid metabolism:** Modulation of lipid metabolism by CLA may be an important mechanism by which CLA exerts its anti-cancer, anti-inflammatory and anti-steatosis effects. The backbone structure of CLA is very similar to oleic acid and therefore, CLA is absorbed and incorporated into lipids in a manner similar to oleic acid (135). CLA is incorporated preferentially into neutral lipids and is found in higher concentrations in the adipose and mammary tissues than liver and plasma (136). However, in vivo and in vitro studies show that c9t11- and t10c12-CLA isomers are also incorporated into membrane phospholipids thereby altering fatty acid metabolism (137). After absorption, CLA is converted to conjugated 18:3 and conjugated 20:3 and 20:4 by delta 6 desaturase and elongases (6; 97). Feeding studies in rats show that while CLA supplementation does not interfere with linoleic acid (LA) retention, the content of linoleic acid metabolites are decreased by increasing doses of CLA (136). Interestingly, these changes in metabolites are specifically observed in adipose tissue and mammary gland. CLA supplementation results in replacement of 20:4 and 20:3 PUFA, substrates for COX and LPOX pathways with conjugated dienes 18:3 and 20:3 by competing with LA, a substrate for delta 6 desaturase resulting in decreased production of eicosanoids (136; 137).

It is now well recognized that the c9t10- and the t10c12-CLA isomers have distinct actions in different tissues. Theses differences may be attributed to differences in their metabolism. The t10c12 isomer is preferentially metabolized in the peroxisomes to a shorter 16:2 conjugated metabolite. This accounts for its lower accumulation into cellular lipids compared to the c9t11 isomer which is metabolized mainly to a conjugated 20:3 isomer (137).
In the liver, CLA decreases hepatic SCD-1 activity and SCD-1 index resulting in an increase in the ratio of saturated:monounsaturated fatty acids, particularly, palmitate:palmitoleate (16:0/16:1) and stearate:oleate (18:0/18:1). These effects may have an impact on lowering TG in the livers of rats supplemented with CLA (138; 139). Similar findings have been reported in mice (140), pigs (141), human pre-adipocytes (142), HepG2 cells (143) and 3T3-L1 adipocytes (144).

**Modulation of genes by CLA:** Several mechanisms have been proposed to explain the effects of CLA on adiposity, diabetes and hepatic steatosis in different species. It seems conclusive that the anti-adiposity effects of CLA are mainly due to the t10c12-CLA isomer (119). CLA exerts its de-lipidative effects in the adipose mainly by increasing genes associated with apoptosis, decreasing fatty acid uptake into adipocytes, increasing energy expenditure, increasing lipolysis, and inducing inflammation, as well as decreasing stromal vascular (SV) cell differentiation (Figure 2.6, p.44) (6). Supplementation with CLA increases uncoupling proteins (UCPs) in the adipose tissue resulting in increased energy expenditure (109; 116; 145). In addition to increasing energy expenditure, CLA, particularly, the t10c12 isomer also induces apoptosis and inhibits differentiation of pre-adipocytes (116; 142; 146). In contrast, CLA reduces adipocyte size without a reduction in number in rats (147). Furthermore, there is increasing evidence showing that the t10c12-CLA isomer increases expression of the inflammatory cytokines TNF-α and IL-6 in the adipose tissue (120; 148). A recent study by Poirier et al. (120) demonstrated that short term administration of t10c12-CLA isomer significantly increased pro inflammatory IL-6, TNF-α and SOCS3 mRNA and significantly decreased PPAR-γ mRNA in the adipose tissue of mice. Furthermore, they
demonstrated a significant induction of macrophage infiltration and induction of apoptosis in the white adipose tissue. The induction of IL-6 was shown to be dependent on activation of NF-κB with the t10c12-CLA isomer in 3T3-L1 adipocytes. Similar results have been previously demonstrated by Brown et al. (149) using human differentiated adipocytes, SV cells, non differentiated SV cells and human adipose tissue explants. They demonstrated that the t10c12-CLA isomer induced insulin resistance in adipocytes by increasing the secretion of inflammatory cytokines, decreasing GLUT-4 translocation to the plasma membrane, and thereby decreasing glucose uptake. These effects were attenuated when NF-κB activation was inhibited using specific inhibitors and si RNA. The t10c12-CLA isomer has also been shown to inhibit adipose tissue proliferation and differentiation at least in part by decreasing PPAR-γ expression. Additionally, CLA decreases the expression of other genes, e.g., CCAAT/enhancer binding protein-a, PPAR-γ responsive genes AP2, perilipin and acyl CoA binding protein that are required for differentiation of pre-adipocytes (142; 150; 151). Available data on the effect of CLA on lipolysis is conflicting. Several studies report decreases in mRNA level of LPL and LPL activity (6) (Table 2.1, p.47). Taken together, these data demonstrate that the t10c12-CLA isomer exerts its anti-adiposity effects by modulating both cellular metabolism and cell cycle control.
Figure 2.6 Mechanisms of de-lipidative effects of t10c12 CLA on adipocytes

(6)
In the liver, CLA feeding produces markedly different responses in rats and mice. In rats, MI-CLA has been shown to decrease hepatic TG accumulation in the ZDF (152), Zucker fa/fa rat (111), and OLETF rat models (114). Further, the t10c12, but not the c9t11-CLA isomer, has been shown to decrease liver TG in OLETF rats (112). In contrast, t10c12-CLA isomer increases hepatic steatosis in association with a rapid depletion of adipose tissue in mice (115; 116; 119). These differences in species have been attributed to rapid depletion of insulin sensitizing adipokines leptin and adiponectin in mice which may be secondary to induction of inflammation in the white adipose tissue with the t10c12-CLA isomer (118; 120).

CLA is a high affinity ligand and activator of peroxisome proliferator-activated receptor (PPAR-α) (153). CLA shares structural similarities with peroxisome proliferators and induces several PPAR-α responsive genes such as liver fatty acid binding protein (L-FABP), acetyl CoA oxidase (AOX), CYP4A1 in vivo and in vitro (153; 154). CLA decreases hepatic TG in conjunction with increases in mRNA and activities of lipid oxidation genes CPT-I and II, AOX, and decreases in lipid synthetic genes FAS and SREBP-1(111; 112). In contrast, CLA has been shown to increase lipid synthetic genes, e.g. FAS, ACC and malic enzyme, in mice (115; 122). Roche et al. (115) demonstrated that the t10c12, but not the c9t11-CLA isomer induced significant hepatic steatosis in ob/ob mice. While the c9t11 CLA isomer significantly decreased hepatic SREBP-1 mRNA and protein, the t10c12 isomer had no effect on this gene. Studies by Degrace et al. (155) demonstrate 100–200% increases in liver CPT I and CPT II isoforms with t10c12-CLA supplementation compared to control fed mice. Together, these studies demonstrate that hepatic steatosis induced by CLA in mice is not due to impaired fatty
acid oxidation. The possibility that CLA increases lipid synthesis and storage requires further investigation. Studies using PPAR-α knock out mice demonstrated that CLA significantly increased lipid oxidative and fatty acid binding genes (156) suggesting that CLA may modulate genes independently of PPAR-α and there may be multiple mechanisms by which CLA exerts its effects. Table 2.1 summarizes changes in gene expression mediated by CLA in the adipose tissue and liver.
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(Table 2.1 continued)
Table 2.1 continued

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Table 2.1 Genes regulated by CLA in the liver and adipose tissue (6).
2.7.2 Flavonoid Naringenin

i. Structure, distribution

Flavonoids are a group of polyphenolic compounds with a characteristic benzo-γ-pyrone structure. They are found in foods of plant origin (157) and are synthesized by secondary metabolism in plants. The basic structure of flavonoids consists of two aromatic rings linked with three carbons (Figure 2.7 A, p.51). The A ring is synthesized by the acetate pathway and has a characteristic hydroxylation pattern at positions 5 and 7. The B ring is synthesized by the shikimate pathway and is hydroxylated at 4’,3’4’ or 3’4’5’ positions (157). Flavonoids can be subdivided further into flavonols, flavanones, flavones, isoflavones, catechins and anthocyanidins based on modifications in the C-ring (Figure 2.7 B, p.51). Over 4000 different flavonoids have been identified and are mainly found in nature as glycosides i.e., conjugated to a sugar moiety making them water soluble (157; 158). The first observation regarding flavonoid biological activities was published by Rusznyak and Szent-Gyorgyi in 1936. The daily intake of flavonoids is estimated to be approximately 0.5-1g (159) and the intakes of flavanones, flavones and flavonols is estimated to be ~23-28 mg/day (157).

There is increasing interest in flavonoid research as several epidemiological studies show possible beneficial effects of flavonoids on cardiovascular diseases and cancer (157; 160). Epidemiological studies also indicate a possible protective relationship between intake of citrus fruits and juices and the risk of lung cancer and ischemic stroke (161-163). In the U.S., the mean daily intake of citrus fruits in individuals has been estimated to be 68g, of which 59g is consumed as juices (U.S. Department of Agriculture 1997). The most commonly consumed citrus juices are orange and grapefruit are rich in
the flavanones naringenin and hesperetin. These citrus flavonoids have been reported to have anti-atherogenic (164-166), anti-carcinogenic (167-169), anti-oxidant (170) and, more recently, anti-diabetic properties (171; 172).
Figure 2.7 (A) Basic structure of flavonoids; (B) Sub-classes of flavonoids
ii. Biological effects

Naringin and naringenin are derived from citrus fruits such as oranges, tomatoes and grapefruit (78mg/100g; approximately 200mg naringenin can be obtained from one medium sized grapefruit) (173). Naringenin is the aglycone form of naringin and is the predominant flavanone in grapefruits and tomatoes (165). Oura et al. (174) first demonstrated that a single injection of 7-O-b naringenin (glucoside form) into streptozotocin induced diabetic rats decreased triglycerides, blood glucose and cholesterol. Recently, the hypoglycemic effects of naringin (glucoside) were demonstrated in db/db diabetic mice (which lack functional leptin receptors). Mice were fed a diet containing 0.2g naringin /kg diet for 5 weeks. Compared with mice fed control diet, mice fed diet with naringin had significantly lower blood glucose and significantly higher liver glycogen levels. Furthermore, mice fed naringin had significantly lower activities of key gluconeogenic enzymes and higher glucokinase activity (171; 172). They concluded that naringin prevented progression of hyperglycemia in db/db mouse model of diabetes by increasing glycolysis and glycogen synthesis and decreasing gluconeogenesis. In a subsequent study, Choi et al. (175) showed that in addition to glucose lowering effects, naringin significantly decreased hepatic TG and plasma cholesterol levels in C57BL/KsJ-db/db mice. These effects were attributed to decreased expression of the lipogenic gene FAS and decreased activity of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase in Sprague Dawley rats fed a high-fat and high-cholesterol diet (176). In addition, naringenin lowers hepatic apoB 100 secretion and decreases hepatic microsomal triglyceride transport protein in vitro (165; 166; 177). Naringenin appeared to act in a similar manner to insulin by increasing the expression of
LDL-r in HepG2 cells. Furthermore, these effects of naringenin were attributed to activation of PI3kinase, (an upstream molecule in the insulin signaling pathway) independent of insulin receptor substrate-1 (IRS-1) resulting in PI3K mediated increase of both cytoplasmic and nuclear SREBP1c levels in HepG2 cells (165). The hypolipidemic effects of naringenin seem to be better understood than its role in preventing hyperglycemia and few in vivo studies have been repeated.

iii. Bioavailability

Several studies have demonstrated that flavonoids naringin and hesperetin which exist mainly as glycosides are hydrolyzed in the gastrointestinal tract before absorption into the gut (178; 179). Felgines et al. (158) compared the absorption of the aglycone naringenin with glycosides (naringenin-7-glucoside and naringenin-7-rhamnoglucoside) after a single administration in a meal in rats. They reported that naringenin was efficiently absorbed in rats, although, the bioavailability differed with the glycoside moiety attached. Furthermore, the major metabolites in the plasma were glucurono- and sulfo-conjugated derivatives of naringenin. Plasma concentrations reached ~ 128 ± 2 µM with naringenin after 10hr. The bioavailability of naringin in humans has also been explored (160). Bioavailability of naringenin was determined in eight human volunteers after consumption of grapefruit and orange juice containing ~ 1283 µmol/L (349 mg/L) and 151µmol/L (41mg/L), respectively. Plasma naringenin concentrations reached ~ 6.0 + 5.4 µmol/L from grapefruit juice and 0.6 + 0.4 µmol/L from orange juice 24 hr after juice consumption.
Several studies in humans have shown that naringenin reaches measurable concentrations in plasma (157). Though inter-individual variability has been reported to be considerable between subjects, plasma levels can reach considerable levels to exert biological effects.

In summary, it is becoming increasingly clear that CLA has many potential biological effects and the fat reducing effects of CLA have been shown in several species. The role of CLA in diabetes is somewhat less clear and the mechanism by which CLA elicits these effects has not been fully elucidated. The grapefruit flavonoid naringenin also has a potential role in attenuating hyperglycemia. However, before these bioactive compounds can be recommended for humans interventions, their mechanisms of action need to be elucidated.
CHAPTER 3

Conjugated Linoleic Acid Does Not Reduce Body Fat but Decreases Hepatic Steatosis in Adult Wistar Rats

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Abbreviations: AOX, acetyl CoA oxidase; CLA, Conjugated linoleic acid; CPT, carnitine palmitoyl CoA; FAS, fatty acid synthase; FBG, fasting blood glucose; LXR, liver X receptor; NEFA, non-esterified fatty acid; NAFLD, Non-alcoholic fatty liver disease; NL, neutral lipid; PPAR, peroxisome proliferator-activated receptor; PL, phospholipid; SCD, stearoyl CoA desaturase; SREBP, sterol regulatory element binding protein; TG, triglyceride.

*Aparna Purushotham, Gayle E. Shrode, Angela A. Wendel, Li-Fen Liu, Martha A. Belury (Accepted: Journal of Nutritional Biochemistry, 2007).
Conjugated linoleic acid (CLA) reduces hepatic lipid accumulation in some rodent models for obesity and hepatic steatosis. However, these effects are variable and complex due to differences in isomer responses and degree and sensitivity to changes in adiposity. Here, we hypothesized that CLA decreases hepatic steatosis in a diet induced model of obesity in rats which are resistant to the adipose lowering effects of CLA. To investigate this, male Wistar rats were fed a high fat (20%) diet for four weeks to induce obesity and hepatic steatosis followed by switching to low fat (6.5%) experimental diets containing either 6.5% soybean oil (CON) or 1.5% CLA triglyceride mix plus 5% soybean oil (CLA). Dietary CLA significantly lowered hepatic triglycerides without changing weight, adiposity or adipokines and was associated with significantly lower hepatic fatty acid synthase and stearoyl CoA desaturase-1 (SCD-1) mRNA levels and SCD-1 index along with significantly lower sterol regulatory element binding protein-1 mRNA, a transcription factor that regulates lipogenesis. Further, the lower lipogenesis was associated with significantly higher mRNA expression of lipid oxidation genes PPAR-α and acetyl CoA oxidase in the livers of rats fed dietary CLA. The lipid lowering effects of CLA in the liver were observed in the absence of changes in adipose tissue and body weight. Thus we conclude that in the Wistar rat model, where adipose levels remain static after feeding dietary CLA, hepatic lipid accumulation is reduced and these effects are not due to an improvement in overall adiposity.
3.2 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) occurs in up to 75% people with obesity and diabetes mellitus (57). NAFLD consists of a spectrum ranging from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH) (57). Hepatic steatosis is the result of excess lipid stored in the liver and may lead to hepatic insulin resistance, glucose intolerance and diabetes (57; 87). Preventing fat accumulation in the livers of mice on a high fat diet has been shown to prevent development of insulin resistance (87).

The group of fatty acids, conjugated linoleic acid (CLA), consists of positional and geometric isomers of octadecadienoate that are naturally found in foods such as meat, milk and other dairy products (95). The isomers, \(c_{9}t_{11}\) and \(t_{10}c_{12}\) are available in commercial oils (e.g. Tonalin™) for human consumption and are most widely studied. It is suggested that \(c_{9}t_{11}\)-CLA and \(t_{10}c_{12}\)-CLA may have differing effects on lipid metabolism in a variety of tissues including liver, muscle and adipose tissue (6; 146). Studies in rat models have shown that CLA attenuates development of hepatic steatosis (111; 113; 152), improves glucose homeostasis and insulin stimulated glucose uptake in the skeletal muscle of the Zucker diabetic fatty (ZDF) rat model for type 2 diabetes (108; 110).

The effects of CLA as both mixed isomers (111; 152) and recently, using individual isomers (112) on attenuation of NAFLD and insulin resistance have been demonstrated only in genetically obese rats. In these studies, CLA improves insulin resistance and
lowers hepatic triglyceride (TG) levels in association with significant depletion of adipose tissue mass (109; 111; 112). For instance, the $t_{10}c_{12}$ isomer decreases hepatic TG, but also decreases adiposity; in contrast, the $c_9t_{11}$ CLA isomer has no effect on either parameter (112). Thus, it is unclear if the improvement in insulin sensitivity and hepatic triglycerides is a result of decreased adiposity. Therefore, we evaluated the effects of CLA in Wistar rats, a useful model to study hepatic steatosis (180; 181) and a diet-induced obesity model which is less susceptible to changes in adiposity associated with dietary CLA (182).

In an effort to understand the mechanism by which CLA modulates steatosis in rodent models, we and others have postulated that CLA isomers alter the progression of NAFLD, in part, by modulating the activity of peroxisome proliferator-activated receptors (PPARs) thereby regulating genetic markers of β-oxidation (153; 183). Further, because the $t_{10}c_{12}$ CLA isomer was recently shown to decrease sterol regulatory element binding protein (SREBP-1) mRNA expression in Otsuka Long Evans Tokushima Fatty (OLETF) rats (112), we postulated that CLA lowers hepatic lipids by suppressing SREBP-1, a transcription factor from the basic helix loop helix leucine zipper family that stimulates lipid synthesis by regulating lipogenic genes such as fatty acid synthase (FAS) and stearoyl CoA desaturase (SCD-1) (115).

Therefore, we evaluated the effects of CLA in Wistar rats, a diet-induced obesity model that is less susceptible to changes in adiposity by dietary CLA (182). The aims of the present study were to: 1) Determine the effect of feeding CLA on attenuation of hepatic steatosis in Wistar rats (fed a high fat diet prior to CLA supplementation); and 2) Explore the molecular mechanisms involved in the process.
3.3 MATERIALS AND METHODS

Materials:

All the diet components were purchased from Bio-Serv (Frenchtown, NJ). CLA mixed triglycerides were obtained from Cognis (Cincinnati, OH). Insulin and leptin Multiplex ELISA kits were obtained from LINCO Research (St. Charles, MO). The rat adiponectin ELISA kit was purchased from B-Bridge International, Inc. (Sunnyvale, CA). The methyl ester standards, PUFA-3 and C:17 FA were purchased from Matreya Inc. (Pleasant Gap, PA). All solvents and other chemicals were of the highest grade commercially available.

Animals:

Four-week-old male Wistar rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA) and were housed 2/cage at 22 °C +/- 0.5 °C on a 12hr day/night cycle. Rats received standard rat chow for two weeks while adjusting to their new environment. All procedures were in accordance with institution guidelines and approved by the Institutional Animal Care and Use Committee of The Ohio State University.

Experimental diets:

Because the objective of this study was to observe the impact of feeding a CLA-rich diet on hepatic steatosis, rats were fed ad libitum a high energy diet, which has previously been shown to induce steatosis in Wistar rats (181) consisting of 20% fat (predominantly lard), 42% carbohydrates and 21% protein (by weight) for four weeks.
prior to assignment to the experimental diets to induce significant gains in hepatic lipids. After four weeks on the high energy diets, rats were randomized by body weight to one of the two isocaloric diets containing 6.5% fat. The diets contained either 6.5% soybean oil (CON diet) or 5% soybean oil with 1.5% CLA triglyceride mix (CLA diet) by weight (Table 3.1, p.64). CLA composition in our diet was a mixture of the two CLA isomers (39.2% c9t11 and 38.5% t10c12 CLA) which contributed approximately 0.5775% (weight of diet) of the adipose-lowering isomer, t10c12-CLA. All diets were modified forms of the AIN-93G diet (184). Rats had free access to food and water. Food intake was measured every other day and body weights were measured weekly.

**Fasting blood glucose:**

After three weeks on 6.5% experimental diets, rats were fasted for 15 hrs and blood was collected from tail vein for analysis of fasting blood glucose using a One Touch Basic glucose analyzer (Lifescan, CA). Insulin sensitivity index was calculated using the fasting serum insulin (FSI) and fasting blood glucose (FBG) with the quantitative insulin sensitivity check index (QUICKI), using the following formula: 

\[ 1/(\log(10) \text{FSI} + \log(10) \text{FBG}) \] (185).

**Necropsy:**

After four weeks on experimental diets, rats were euthanized (after 6hr of fasting) by CO₂ and liver, epididymal adipose, peri-renal and gastrocnemius muscle tissues were weighed, snap-frozen in liquid nitrogen, and stored at -80°C for further analysis. Blood samples were collected in tubes, centrifuged to isolate serum and stored at -80°C for hormone and metabolite analyses.
**Serum hormone and metabolite determination:**

Serum insulin, leptin, and adiponectin levels were determined using ELISAs. Serum triglycerides and non esterified fatty acids (NEFA) were measured using spectrophotometric assays purchased from Sigma (St. Louis, MO) and Wako Chemicals (Richmond, VA) respectively.

**Tissue TG analysis:**

TG from liver and muscle were quantitatively measured with an enzymatic colorimetric kit (Sigma). In short, tissue sections were homogenized and lysed in RIPA buffer (Santa Cruz Biochemicals, CA). Protein was determined using BCA assay (Pierce Biotechnology Inc., IL). TG were extracted with 2:1 (v:v) chloroform:methanol using the Folch method (186) and dissolved in isopropanol. TG were measured using the glycerol determination procedure (Sigma, St. Louis, MO). Values were normalized to tissue protein content.

**Real-time Reverse Transcriptase PCR:**

Liver tissue was homogenized in Trizol reagent (Sigma) and mRNA was isolated using manufacturer’s protocol. RNA was quantified by spectrophotometry and diluted in RNase-free water. RNA integrity was assessed by electrophoresis using agarose gel and ethidium bromide staining. The first transcripts were reverse transcribed using reverse transcriptase (Invitrogen) and cDNA were amplified using real-time PCR with TaqMan gene expression assays (Applied Biosystems, CA). In short, 10ng of the reverse transcription reaction was amplified in a total reaction volume of 25µl using pre-designed, validated FAM labeled primers designed for stearoyl CoA desaturase (SCD-1; NM_139192.1), fatty acid synthase (FAS; NM_017332.1), liver fatty acid binding protein...
(L-FABP; NM_012556.1), acetyl CoA oxidase (AOX; NM_017340.1), sterol regulatory element binding protein (SREBP-1; AF286469), carnitine palmitoyl CoA transferase (CPT I; NM_031559.1 and CPT II; NM_012930.1), phosphoenolpyruvate carboxykinase (PEPCK; NM_198780.3) and glucose-6-phosphatase (G-6-Pase; NM_176077.3) using universal cycling conditions (Applied Biosystems, CA). Target gene expression was normalized to Vic labeled18 s, which was used as an endogenous control in the same reaction as the target gene.

**Liver fatty acid composition:**

Lipids were extracted from livers using the Folch method (186). Briefly, liver tissue was homogenized in methanol containing BHT and lipids were extracted in chloroform:methanol (2:1, v:v). Heptadecanoic acid was used as an internal control. Extracted fatty acids from each rat were dissolved in methyl tert-butyl ether (MTBE): acetic acid (100:0.2, v/v) and further separated into neutral and phospholipids using a silica Sep-Pak column procedure, as described previously (187). Fatty acids were methylated by incubating with tetramethylguanidine in a 100°C water bath, and centrifuging in 0.88% KCl:hexane (1:2) at 1000 x g for 10 minutes. Fatty acid methyl esters were analyzed by gas chromatography (HP 5890 equipped with FID and 30-m Omegawax™ capillary column, Supelco Chromatography Products). Fatty acids were identified using authentic standards (Matreya Inc, Pleasant Gap, PA), quantified by determining areas under identified peaks (ChemStation Software; Packard Instrument Company, Meriden, CT), and adjusted to the internal control. SCD-1 activity index was calculated from product-to-precursor ratios as follows: palmioleate to palmitate (16:1n-7/16:0) and oleate to stearate (18:1n-9/18:0) (188).
**Statistical analysis:**

All data are presented as mean $\pm$ SE. Data were analyzed using MINITAB (version 14, State College, PA). Differences between means were analyzed using two sample Student’s $t$ test. Differences were considered significant at P < 0.05.
<table>
<thead>
<tr>
<th>(g/kg)</th>
<th>High fat (20%)</th>
<th>Low fat (6.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>CLA</td>
</tr>
<tr>
<td>Starch</td>
<td>333.39</td>
<td>402.49</td>
</tr>
<tr>
<td>Casein</td>
<td>234.10</td>
<td>200.00</td>
</tr>
<tr>
<td>Cystine</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Choline bit</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Salt mix</td>
<td>35.00</td>
<td>35.00</td>
</tr>
<tr>
<td>Lard</td>
<td>190.00</td>
<td>--</td>
</tr>
<tr>
<td>Sucrose</td>
<td>--</td>
<td>100.00</td>
</tr>
<tr>
<td>Lo Dex</td>
<td>132.00</td>
<td>132.00</td>
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<tr>
<td>Fiber</td>
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</tr>
<tr>
<td>Tbhq</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>10.00</td>
<td>65.00</td>
</tr>
<tr>
<td>CLA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1000.00</strong></td>
<td><strong>1000.00</strong></td>
</tr>
</tbody>
</table>

Table 3.1 Diet composition
3.4 RESULTS

*Body weights, food intake and organ weights:*

After feeding a high fat (20% fat) diet for four weeks, rats were fed experimental diets containing 6.5% soybean oil (CON) or 5% soybean oil plus 1.5% CLA for four additional weeks. Switching to low fat diets did not result in weight loss in Wistar rats ([Figure 3.1](#)). Diet containing CLA did not change either body weights or food intake significantly compared to diet without CLA (CON). In addition, there were no significant differences in the weights of liver, epididymal or peri-renal adipose tissue between the two diet groups ([Table 3.2, p.68](#)) after four weeks on the experimental diets.
Figure 3.1 Body weight over time in Wistar rats. Rats were fed a high fat diet (20% fat by weight) for four weeks followed by switching to low fat (6.5 %fat) experimental diets for additional four weeks. The diets contained either 6.5% soybean oil (CON diet, closed circles) or 5% soybean oil with 1.5% CLA triglyceride mix (CLA diet, open circles). Values represent mean ±SE of 8 rats per group.
**Plasma concentration of metabolites:**

Because dietary CLA modulates metabolism which may be responsive to insulin and adipokines (111; 116; 118), we measured leptin and adiponectin levels post-necropsy (rats were fasted for 6 hr) after four weeks on the experimental diets. Additionally, we measured fasting glucose levels after an overnight fast following three weeks of feeding experimental diets. Although rats fed neither CON nor CLA diet developed hyperglycemia in this study, feeding dietary CLA decreased fasting glucose levels significantly compared to rats fed the CON diet (Table 3.2). The lower fasting glucose levels were not accompanied by changes in circulating concentrations of insulin or leptin. While CLA did not increase adiponectin levels significantly compared to rats fed the CON diet, there was a higher trend of this insulin-sensitizing adipokine in the CLA group (P=0.058). We measured insulin sensitivity in Wistar rats using the QUICKI index. Insulin sensitivity was not different between rats fed CON and CLA diet (Table 3.2).

**Liver Triglycerides:**

Dietary CLA significantly decreased TG accumulation in the liver by approximately 20% (Table 3.2) compared to rats fed the CON diet. However, muscle and serum TG levels did not differ significantly between the two groups (Table 3.4, appendix, p.). Because adipose tissue can contribute to a significant amount of lipid influx to the liver by lipolysis from adipose tissue, we measured serum non-esterified fatty acids (NEFA) concentrations. Serum NEFA concentrations were unchanged by feeding the diet containing CLA for four weeks (Table A.1, p.171) which corroborate with the lack of change in adipose tissue mass in Wistar rats (Table 3.2).
Table 3.2 Body weights and serum metabolites

*Significantly different from CON group, *p<0.05. Values represent mean ±SE of 16 rats per group. § n=8 rats per group.
Liver mRNA expression:

Next, to understand the mechanism by which dietary CLA decreased hepatic TG levels in the absence of a reduction in body weight, we measured the mRNA levels of genes that modulate lipid metabolism in the liver using real-time PCR. Dietary CLA significantly increased mRNA levels of both PPAR-α and AOX. The mRNA level of L-FABP was higher in the CLA-fed rats but not significantly different compared to rats fed the CON diet (Figure 3.2 A-C). mRNA levels of CPT-I and CPT-II, key enzymes in mitochondrial β-oxidation, did not change with diet containing CLA (data not shown). Dietary CLA decreased SCD-1 transcript levels by 80% compared to rats fed CON diet (Figure 3.2 D), and significantly decreased FAS expression (53%) (Figure 3.2 E). Associated with lower expression of lipogenic genes FAS and SCD-1, mRNA levels of SREBP-1 were significantly decreased in rats fed the CLA diet (Figure 3.2 F).
Figure 3.2 Effect of CLA on hepatic mRNA expression of genes involved in lipid metabolism. Rats were fed a high fat diet (20% fat by weight) for four weeks followed by switching to low fat (6.5 %fat) experimental diets for additional four weeks. The diets contained either 6.5% soybean oil (CON diet) or 5% soybean oil with 1.5% CLA triglyceride mix (CLA diet). *(A)* PPAR-α mRNA; *(B)* AOX mRNA; *(C)* L-FABP mRNA; *(D)* SCD-1 mRNA; *(E)* FAS mRNA; *(F)* SREBP-1 mRNA. Values represent mean ± SE of 8 rats per group, * represent significant differences between groups, p<0.05.
A. Diet

B. Diet

C. Diet

D. Diet

E. Diet

F. Diet
**Hepatic SCD-1 index:**

To understand the liver-specific effects of dietary CLA, we analyzed the liver fatty acid composition in different lipid fractions using gas chromatography. Levels of both c9t11 and t10c12 isomers were significantly higher in liver neutral and phospholipid fractions of rats fed CLA compared to rats fed the CON diet (Table 3.3). Because mRNA levels of SCD-1 were dramatically decreased by dietary CLA, we examined the index of SCD-1 activity. The SCD-1 index was quantified by determining the amount of conversion of saturated (18:0 and 16:0) fatty acids to monounsaturated (18:1 and 16:1) fatty acids. The ratios of palmitoleic acid to palmitic acid (16:1/16:0) and oleic acid to stearic acid (18:1/18:0) serve as surrogate markers for SCD-1 activity as these fatty acids are the predominant substrates for SCD-1 enzyme (189). Liver SCD-1 indices for the different fatty acids in the two pools of fatty acids are shown in Figure 3.3. In comparison with rats fed CON diet, rats that were fed the diet containing CLA had significantly lower SCD-1 indices in both lipid fractions for the two fatty acids. Supplementation with CLA resulted in approximately 40% and 26% reduction in the SCD-1 index for neutral and phospholipid fractions, respectively. This suggests that CLA lowered liver TG by not only decreasing mRNA levels but also by decreasing activity as suggested by the SCD-1 indices.
<table>
<thead>
<tr>
<th>Fatty acid (%)</th>
<th>Liver NL</th>
<th></th>
<th>Liver PL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>CLA</td>
<td>p-value</td>
<td>CON</td>
</tr>
<tr>
<td>16:0</td>
<td>26.38 ± 0.78</td>
<td>26.54 ± 0.63</td>
<td>0.872</td>
<td>17.95 ± 0.54</td>
</tr>
<tr>
<td>16:1</td>
<td>6.50 ± 0.74</td>
<td>3.91 ± 0.43</td>
<td>0.013*</td>
<td>1.37 ± 0.16</td>
</tr>
<tr>
<td>18:0</td>
<td>2.33 ± 0.19</td>
<td>3.15 ± 0.19</td>
<td>0.011*</td>
<td>20.32 ± 0.83</td>
</tr>
<tr>
<td>18:1 n-9</td>
<td>27.65 ± 0.76</td>
<td>26.58 ± 1.11</td>
<td>0.445</td>
<td>3.64 ± 0.11</td>
</tr>
<tr>
<td>18:1 n-7</td>
<td>5.47 ± 0.47</td>
<td>4.62 ± 0.37</td>
<td>0.182</td>
<td>4.85 ± 0.38</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>26.45 ± 1.25</td>
<td>24.35 ± 1.64</td>
<td>0.332</td>
<td>13.45 ± 0.48</td>
</tr>
<tr>
<td>c9t11 CLA</td>
<td>1.82 ± 1.04</td>
<td>6.54 ± 1.86</td>
<td>0.000*</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>t10c12 CLA</td>
<td>0.00 ± 0.00</td>
<td>1.04 ± 0.05</td>
<td>0.000*</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>20:4</td>
<td>2.68 ± 0.22</td>
<td>2.53 ± 0.37</td>
<td>0.719</td>
<td>29.79 ± 0.39</td>
</tr>
<tr>
<td>22:6</td>
<td>0.74 ± 0.21</td>
<td>0.75 ± 0.23</td>
<td>0.968</td>
<td>8.51 ± 0.34</td>
</tr>
</tbody>
</table>

Table 3.3 Liver fatty acid composition

*Significantly different from CON group, *p<0.05. Values represent mean ± SE of 8 rats per group.
Figure 3.3  Effect of CLA on hepatic SCD-1 index. Liver SCD-1 index was measured by measuring the SCD-1 index in neutral (NL) and phospholipid (PL) fractions using gas chromatography. SCD-1 index was calculated for the two predominant substrates palmitate and stearate.  

**A-B**: Neutral lipids fraction.  
**C-D**: Phospholipid fraction. Values represent mean ± SE of 8 rats per group, * represent significant differences between groups, p<0.05.

Liver SCD-1 index was measured by measuring the SCD-1 index in neutral (NL) and phospholipid (PL) fractions using gas chromatography. SCD-1 index was calculated for the two predominant substrates palmitate and stearate. **A-B**: Neutral lipids fraction. **C-D**: Phospholipid fraction. Values represent mean ± SE of 8 rats per group, * represent significant differences between groups, p<0.05.
3.5 DISCUSSION

The impact of dietary CLA on hepatic steatosis and insulin sensitivity is controversial due to differences in species, isomer responses and degree and sensitivity to changes in adiposity (111; 112; 115; 190). Here, we investigated the effects of dietary CLA on attenuation of hepatic steatosis in male Wistar rats, a rodent model for hepatic steatosis that are less responsive to changes in adiposity induced by CLA (182). Rats were fed a high-fat diet for four weeks prior to CLA supplementation (as part of a low fat diet) to induce hepatic steatosis (180; 181). While rats did not lose body weight when switched to low fat experimental diets, feeding CLA as part of a low fat diet to adult Wistar rats for four weeks significantly decreased hepatic lipid accumulation compared to rats fed low fat diet without CLA (CON diet). Further, these changes in hepatic TG were observed in the absence of changes in body weight, epididymal and peri-renal adipose tissue or food intake. These results may be in accordance with a previous study in Wistar rats where CLA supplementation increased adipose tissue turnover without altering adipose mass (182). While rats fed the CON diet developed hepatic steatosis (5.7%), which is defined as accumulation of lipids greater than 5% of liver weight (191), the livers of rats fed dietary CLA were not steatotic (4.6%). Our results using this diet-induced model of fatty liver are in accordance with other studies showing similar effects in the livers of genetically obese rat models (111-113).
In contrast to studies showing changes in serum lipid concentrations (109; 115) and muscle in ZDF rats (109), we did not observe any significant differences in muscle and serum TG or NEFA with feeding CLA to Wistar rats. The absence of changes in serum TG and NEFA in the present study indicates that the reduction of hepatic steatosis by dietary CLA could be due to the action of CLA to modulate lipid metabolism in the liver. Prior studies using various rat models have shown that CLA supplementation is associated with alterations in more than a particular fat depot (109; 112; 192). In the present study, the reduction in hepatic TG by CLA occurred in the absence of changes in weight and at least two depots of adipose mass; epididymal and peri-renal adipose mass. These results suggest that in the present study, the attenuation of liver TG was not a consequence of overall improvement in adiposity.

CLA regulates lipid metabolism in various tissues by modulating lipid oxidation (111; 153; 193), lipolysis (111; 146) and de novo lipogenesis (116; 140; 194). We measured PPAR-α responsive genes in the liver (e.g., L-FABP, CPT-1 and AOX-1) as a possible mechanism contributing to lower lipids in the livers of rats fed the CLA diet. In Wistar rats, CLA significantly increased the mRNA levels of PPAR-α and AOX, suggesting increased peroxisomal oxidation. These results are in accordance with data suggesting that reduced expression of PPAR-α and AOX, a key enzyme which regulates peroxisomal β-oxidation, are associated with development of NAFLD in OLETF rats (195). Further, our data showing unchanged mRNA levels of CPT-I and CPT-II, key enzymes involved in mitochondrial β-oxidation support findings by others using human fibroblasts, which demonstrate that NAFLD is not associated with reduced mitochondrial β-oxidation (59). In contrast to these findings, other studies have shown that CLA
feeding is associated with unchanged mRNA levels but higher CPT-I activity in obese rats along with improvement in hepatic steatosis (111; 112). The higher expression of lipid oxidation genes in the livers of rats fed dietary CLA was accompanied by significantly lower lipogenic gene FAS mRNA, similar to findings in a recent study conducted in the OLETF rat model using 1% \( t_{10c12} \) CLA isomer (112). In the present study, mixed isomers of CLA (providing approximately 0.6% of the \( t_{10c12} \) CLA isomer) had similar effects on lowering hepatic TG in adult Wistar rats.

One mechanism by which CLA modulates lipid synthesis and accumulation is by decreasing SCD-1 mRNA and activity (138; 140; 144). SCD-1 catalyzes the rate-limiting step in the cellular biosynthesis of monounsaturated fatty acids, primarily oleate (18:1) and palmitoleate (16:1) from stearic (18:0) and palmitic (16:0) acids. Oleate and palmitoleate can be incorporated into and stored as TG in the liver (196). Additionally, inhibition of SCD-1 is associated with increased \( \beta \)-oxidation (197), as demonstrated in SCD-1 knockout mice, which are protected against diet- and leptin deficiency-induced adiposity and hepatic steatosis. In the present study, CLA significantly decreased both mRNA expression and index of hepatic SCD-1 compared to CON animals. Our results are in accordance with studies showing similar effects of CLA on SCD-1 (140; 144). However, the effect of CLA on TG accumulation was not determined in these prior studies. While the effects of CLA on adiposity have been shown to be independent of SCD-1, the \( t_{10c12} \) CLA isomer failed to promote hepatic steatosis in SCD-1 knockout mice (198), suggesting that some of the effects of CLA on hepatic lipid metabolism are modulated by SCD-1.
In the present study, the hepatic lipid-lowering effect of CLA may be attributed at least in part to suppression of SCD-1 and FAS. The precise mechanism by which CLA regulates SCD-1 is still unknown. However, it has been postulated that CLA inhibits SCD-1 by binding to the active site or to an unidentified allosteric site on the SCD-1 enzyme (138). Lipogenic genes such as FAS are regulated by SREBP-1. In addition, PUFA have been shown to modulate SCD-1 by direct inhibition of SREBP-1 transcription by disrupting liver X receptor (LXR) action (199). In the present study, we saw a significant suppression of SREBP-1 mRNA by CLA, which may be responsible for the decrease in lipogenic genes observed in this study. We further show that the effects of CLA on SCD-1 in this study were independent of the adipokine leptin, a known SCD-1 suppressor, which was unchanged in rats fed CLA.

Hepatic steatosis is closely associated with decreased sensitivity of the liver to insulin, which may lead to hyperglycemia and hyperinsulinaemia. In the present study, rats fed neither CON nor CLA diet developed significant hyperglycemia. In fact, rats fed a CLA enriched diet had significantly lower fasting glucose levels but was not accompanied with changes in mRNA levels of glucose metabolizing genes (e.g. PEPCK, G-6-Pase- data not shown). We also did not see a change in insulin sensitivity calculated using QUICKI. We chose to study the effects of CLA in the Wistar rat model, which is known to be sensitive to hepatic steatosis, but are not a typical model to study hyperglycemic conditions such as diabetes and metabolic syndrome.

Hormones secreted from the adipose tissue, such as leptin, adiponectin and tumor necrosis factor-α (TNF-α), have a profound impact on steatosis and insulin sensitivity (7; 116; 200-202). Higher circulating concentrations of adiponectin are inversely associated
with insulin resistance and hepatic steatosis by increasing fatty acid oxidation along with reduction of hepatic gluconeogenesis and increased glucose uptake by the skeletal muscle (40). Previously, it has been shown that CLA feeding attenuates hepatic steatosis and improves hyperglycemia in ZDF rats in association with lower TNF-α mRNA levels and increased in adiponectin levels (111). Studies that report induction of insulin resistance and NAFLD by CLA have shown that CLA causes a sharp decline in circulating levels of adiponectin even before depletion of adipose tissue occurs (118). In the present study, we saw a modest but not significant increase in serum adiponectin levels with CLA supplementation.

In conclusion, our data suggest that CLA lowers hepatic lipids in adult Wistar rats despite no changes in body weight and epididymal or peri-renal adipose tissue. Therefore, it appears that in Wistar rats, which are induced to be obese by diet (and not genetic manipulation), the effect of CLA on lowering hepatic triglycerides was not likely a result of an overall improvement in adiposity. Further, the effects of CLA in adult Wistar rats occur when adipokines (e.g. leptin and adiponectin) are maintained suggesting that in the presence of sufficient adipose and/or adipokines, CLA attenuated hepatic steatosis by modulating hepatic genes involved in lipogenesis and lipid oxidation. The varied effects of CLA on hepatic steatosis and insulin sensitivity in numerous animal models for obesity may depend on differences in fat-to-lean partitioning between adipose and liver tissues.
3.6 ACKNOWLEDGMENTS

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CHAPTER 4

Maintenance of adiponectin attenuates insulin resistance induced by dietary conjugated linoleic acid in mice

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*Abbreviations: Acetyl CoA oxidase (AOX), area under the curve (AUC), conjugated linoleic acid (CLA), fasting blood glucose (FBG), fatty acid synthase (FAS), fatty acid transporter (CD36), insulin resistance (IR), insulin tolerance test (ITT), non-esterified fatty acid (NEFA), rosiglitazone (ROSI), triglyceride (TG), and tumor necrosis factor (TNF-α).

4.1 ABSTRACT

Conjugated linoleic acid (CLA) causes insulin resistance and hepatic steatosis in conjunction with depletion of adipokines in some rodent models. Our objective was to determine if maintenance of adipokines mainly leptin and adiponectin by either removing CLA from diets or using an adiponectin enhancer, rosiglitazone (ROSI) could attenuate CLA induced insulin resistance. Male C57BL/6 mice were consecutively fed two experimental diets containing 1.5% CLA mixed isomer for four weeks followed by a diet without CLA for four weeks. CLA significantly depleted adiponectin, but not leptin and was accompanied by hepatic steatosis and insulin resistance. These effects were attenuated after switching mice to the diet without CLA along with restoration of adiponectin. To further elucidate the role of adiponectin in CLA mediated insulin resistance, ROSI was used in a subsequent study in male ob/ob mice fed either CON or CLA diet. ROSI maintained significantly higher adiponectin levels in CON and CLA fed mice and prevented depletion of epididymal adipose tissue and development of insulin resistance. In conclusion, we show that insulin resistance induced by CLA may be related more to adiponectin depletion than leptin and maintaining adiponectin levels alone either by removing CLA or using ROSI can attenuate these effects.
4.2 INTRODUCTION

Type 2 diabetes is characterized by impaired glucose and lipid metabolism and is associated with obesity (203). Adipose tissue not only stores excess energy but also has important endocrine functions. Proteins secreted from the adipose tissue, known as adipokines, have important functions in regulating whole body metabolism (204). In particular, the adipokine adiponectin was identified in the adipose tissue and plasma of humans and rodents (205-208) and is inversely associated with obesity and type 2 diabetes (44; 202). Administration of adiponectin attenuates insulin resistance (IR) by decreasing tissue triglyceride (TG) levels as a result of increased fatty acid oxidation (45; 209). In addition, adiponectin lowers hyperglycemia by suppressing hepatic glucose production along with increasing glucose uptake by the skeletal muscle (45; 202).

Conjugated linoleic acid (CLA) consists of positional and geometric isomers of octadecadienoate that are naturally found in foods such as beef, lamb, milk and other dairy products (95). It is well-established that c9t11-CLA and t10c12-CLA have unique effects on lipid metabolism and it is the t10c12-CLA isomer that is mainly associated with decreases in body fat in experimental rodent models (109; 115; 129; 210) as well as in some human studies (132; 211), independently of energy intake (212).

Feeding CLA to mice is associated with lipodystrophy and worsening of insulin sensitivity (115-119). These effects have been attributed to rapid and significant reduction of adipose tissue and a sharp decline in insulin sensitizing adipokines such as
adiponectin and leptin (118). While feeding dietary CLA has been previously shown to deplete adipokines and cause hyperinsulinaemia (118), it is unknown whether removing CLA from the diet can restore the level of adipokines and attenuate insulin resistance showing that there is in fact, a causal link between adipokine depletion by feeding dietary CLA and development of hepatic steatosis and insulin resistance in mice. Further, dietary CLA has been shown to induce insulin resistance in *ob/ob* mice which lack functional leptin (115) raising the possibility that adipokines other than leptin may be more important in CLA mediated insulin resistance in mice. Thus, we further investigated the role of adiponectin in insulin resistance mediated by CLA in leptin deficient *ob/ob* mice. To this end, we used the peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonist rosiglitazone (ROSI) in conjunction with CLA and hypothesized that maintaining serum adiponectin in *ob/ob* mice, which lack functional leptin attenuates the effects of CLA on insulin resistance and hyperglycemia.
4.3 MATERIALS AND METHODS

Materials:

Diet components were purchased from Research Diets (New Brunswick, NJ) and Bio-Serv (Frenchtown, NJ) for studies 1 and 2 respectively. CLA mixed triglycerides (39.2% c9t11 and 38.5% t10c12 CLA) were obtained from Cognis (Cincinnati, OH). Rosiglitazone was obtained from Cayman Chemical (Ann Arbor, MI).

Animals:

Eleven-week old male C57BL/6 mice and six week old male ob/ob mice were purchased from Harlan (Indianapolis, IN) and Charles River Laboratories, Inc. (Wilmington, MA) respectively. Mice were housed 4 per cage at 22 °C +/- 0.5 °C on a 12-hr day/night cycle. Mice received standard chow for one week while adjusting to their new environment. All procedures were in accordance with institution guidelines and approved by the Institutional Animal Care and Use Committee of The Ohio State University.

Study 1 Depletion-repletion of Adipokines:

To determine a role of adipokine depletion by CLA in the development of insulin resistance and hepatic steatosis, 12-week old male C57BL/6 (n=10) were consecutively fed two different experimental diets. For the first 4 weeks, mice were fed 1.5% CLA experimental diet, which contained 5% soybean oil plus 1.5% CLA triglyceride mix by weight for a total of 6.5% fat. This dose of CLA provided approximately 0.6% (by
weight) each of the c9t11 and c10c12 CLA isomers, which have been shown to effectively reduce adipose tissue in rodents (109; 118; 119). At the end of the first 4 week diet period, half of the mice were sacrificed and the remaining mice were switched to a diet without CLA (chow diet containing 4-5% total fat). All mice had free access to food and water. Body weights were measured at indicated time points.

**Study 2 Sustenance of adiponectin:**

Six-week old male ob/ob mice were randomized by body weight and fed experimental diets containing 6.5% total fat for 4 weeks. The diets contained 6.5% soybean oil (CON diet, n=8) by weight. Additionally, ten mice were maintained on CON diet for the first 2 weeks following which six mice were switched to the CLA diet and four mice were continued on the CON diet for the last two weeks of study period. During the last two weeks mice received daily intra-peritoneal (IP) injections of PBS (vehicle control-DMSO 10% and PBS solution 90%) or 10mg/kgBwt/day ROSI (213; 214). Body weights were measured weekly.

**Insulin tolerance test and Fasting blood glucose (FBG):**

Insulin sensitivity was determined for studies 1 and 2 using an insulin tolerance test at indicated times. Mice were fasted overnight and received intraperitoneal injections of insulin, Humulin R (Eli lily Inc) at doses of 0.75U/kg body weight for C57BL/6 and 1.5U/kg body weight for ob/ob mice. Insulin stimulated glucose clearance was determined by tail vein bleeding at 0, 15, 30, 45, 60, 90 and 120 min after insulin injection. Insulin sensitivity was determined by calculating the areas between the curves and individual baselines were used to normalize data.
FBG levels were measured at baseline, two weeks and four weeks for study 2. Mice were fasted overnight for 12 hrs and tail vein blood was used to analyze FBG using a One Touch Basic glucose analyzer (Lifescan, Milpitas, CA).

**Necropsy (study 1 and 2):**

In order to avoid the effects of injected insulin on gene expression, mice were anesthetized by isofluorane in the fasted state 2 days after the insulin tolerance test. Blood was collected by heart puncture, centrifuged at 1500 x g at 4°C to isolate serum and stored at -80°C for hormone and metabolite analyses. Liver, epididymal adipose, and gastrocnemius muscle tissues were weighed, snap-frozen in liquid nitrogen, and stored at -80°C for further analysis.

**Serum hormone and metabolite determination:**

Time course depletion-repletion of adipokines from study 1 were determined using 4-hr fasted serum from retro-orbital eye bleeds at the indicated time points. Mice were anesthetized using isoflurine. Additionally, fasted serum insulin, adiponectin and resistin levels from study 2 were determined using ELISA’s (LINCO Research, St. Charles, MO). Fasting serum triglycerides and non-esterified fatty acids (NEFA) from study 2 were measured using spectrophotometric assays from Sigma (St. Louis, MO) and Wako Chemicals, (Richmond, VA) respectively.

**Tissue TG analysis (study 1 and 2):**

Liver and muscle tissues were homogenized and lysed in 10x Tris (w/v) buffer containing 20mM trizma base, 1% trition-X100, 50mM NaCl, 250mM sucrose, 50mM NaF, 5mM Na₄P₂O₇.10H₂O and protease inhibitors. TG were extracted with 2:1(v/v) chloroform:methanol, final extracts were dissolved in 3:1:1 (v/v/v) tert-
butanol:methanol:triton X-100 (215) and TG were quantitatively measured with an enzymatic colorimetric kit (Sigma). Values were expressed as percentage tissue weight.

**Real-time RT PCR (study 2):**

Sections from liver and muscle tissue were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) and RNA was isolated using the manufacturer’s protocol. RNA from adipose tissue was isolated using the RNeasy lipid extraction kit (Qiagen, Valencia, CA). RNA was diluted in RNase-free water and quantified by spectrophotometry. RNA integrity was assessed by electrophoresis using agarose gel and ethidium bromide staining. The first transcripts were reverse transcribed using reverse transcriptase (Invitrogen) and cDNA was amplified using real-time PCR with FAM labeled TaqMan gene expression assays (Applied Biosystems, Foster City, CA). In short, 5 ng of the reverse transcription reaction was amplified in a total reaction volume of 25µl using pre-designed and validated primers for liver fatty acid synthase (FAS), fatty acid transporter (CD36), and acetyl CoA oxidase (AOX), and tumor necrosis factor (TNF-α) using universal cycling conditions. Target gene expression was normalized to Vic labeled18s, which was used as an endogenous control and amplified in the same reaction as the target gene.
**Statistical analysis:**

All data are presented as mean ± SE. Data were analyzed using MINITAB (version 14, PA). Data from study 2 were analyzed by one-way ANOVA. Post-hoc analysis was performed using Tukey’s test. Other comparisons were analyzed by Student’s t test as appropriate. Weight gain and serum adipokine concentrations over time were analyzed by repeated measures ANOVA using Statistical Analysis System (version 9.1, Cary, NC). Differences were considered significant at P < 0.05.
4.4 RESULTS

Study 1 Body weights and organ weights:

Body weights and weight gain were significantly reduced in C57BL/6 mice after 4 weeks of supplementation with dietary CLA (Table 4.1 and Figure 4.1). Supplementation with dietary CLA significantly decreased epididymal adipose mass and increased liver weight. When CLA was removed from the diets, body weight and adipose tissue mass increased significantly. Concomitant with increased body weight and adipose tissue weight, liver weights significantly decreased after four weeks on the diet without CLA (Table 4.1).
Figure 4.1 Effect of dietary CLA on weight gain from study 1. Male C57BL/6 mice were fed a diet containing 1.5% CLA (+CLA, n=10) for four weeks followed by four weeks without CLA (-CLA, n=5) * P<0.05 vs. baseline. § P<0.05 vs. last time point on the diet containing CLA.
Table 4.1 Body weights and organ weights

Body and organ weights of male C57BL/6 mice at baseline (n=10), after four weeks of feeding diet with +CLA (n=5) and after an additional four weeks of feeding diet without -CLA (n=5). Values represent mean ± SE. Superscripts represent significant differences between treatments. * P<0.05 vs. baseline, § P<0.05 vs. +CLA.
Serum metabolites:

Levels of adiponectin decreased over time in C57BL/6 mice on the diet containing CLA. Significant differences were observed at day 6 and adiponectin levels continued to decrease over-time (Figure 4.2B). Switching mice to the diet without CLA significantly increased adiponectin levels however, levels remained significantly lower (50% of baseline) than baseline in C57BL/6 mice. In contrast, leptin levels were less responsive to dietary CLA and were not significantly different compared to baseline (Figure 4.2A).
Figure 4.2 Effect of CLA on time course depletion/repletion of adipokines from study 1. Male C57BL/6 mice were fed a diet containing 1.5% CLA (+CLA, n=10) for four weeks followed by four weeks without CLA (-CLA, n=5). (A) Serum leptin in male C57BL/6 mice; (B) serum adiponectin in male C57BL/6 mice. Adipokine concentrations were determined at indicated times. * P<0.05 vs. baseline, § P<0.05 vs. last time point on the diet containing CLA.
**Insulin tolerance:**

Insulin sensitivity was significantly worsened in C57BL/6 mice after 4 weeks on the CLA diet (AUC, Table 4.1 and Figure 4.3). Switching mice to the diet without CLA significantly improved insulin sensitivity. The improvement in insulin sensitivity was significant after two weeks following the switch to the diet without CLA.

**Liver TG:**

Corresponding to increased liver weights, 4 weeks of feeding dietary CLA significantly increased hepatic TG in C57BL/6 mice. Switching to the diet without CLA for 4 weeks significantly attenuated hepatic steatosis (Table 4.1).
Figure 4.3 Effect of dietary CLA on insulin sensitivity. Insulin sensitivity was measured at baseline (n=10, closed circles), after four weeks on dietary CLA (+CLA, n=10, open circles) and after two weeks on diet without CLA (-CLA, n=5, closed triangles). Significance was determined using AUC (Table 4.1).
Study 2 Body weights and organ weights:

ROS1 administration for two weeks prevented weight loss in male ob/ob mice fed dietary CLA and weight gain was comparable to CON-PBS mice in both ROS1 treated groups (Figure 4.4). Epididymal adipose mass was also not significantly different in the CLA-ROS1 group compared to the CON-ROS1 and CON-PBS group (Table 4.2).

Serum metabolites:

Two weeks of treatment with ROS1 not only prevented increases in both glucose and insulin in mice fed dietary CLA (CLA-ROS1 vs. CON-ROS1 and CON-PBS) but also significantly decreased FBG levels compared to CON-PBS mice (Table 4.2). ROS1 administration significantly increased adiponectin levels in mice fed the CON or CLA diet compared to mice fed the CON-PBS diet. Serum resistin levels were significantly higher in CON-PBS mice compared to CON-ROS1 mice however; ROS1 administration did not have an effect on resistin levels in mice fed CLA. Further, ROS1 administration significantly decreased serum levels of TG and NEFA in both CON- and CLA- fed mice compared to CON-PBS group (Table 4.2).
Figure 4.4 Effect of dietary CLA on weight gain from study 2. Male ob/ob mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either PBS or ROSI for two weeks by IP injection daily. CON-PBS (n=8, closed circles), CON-ROSI (n=4, open circles), CLA-ROSI (n=6, closed triangles). * P<0.05.
Table 4.2 Body weights and serum metabolites

Male ob/ob mice were fed experimental diets containing 6.5% soybean oil (CON diet, n=8) for four weeks. Additionally, ten mice were maintained on CON diet for the first 2 weeks following which six mice were switched to the CLA diet containing 5% soybean oil plus 1.5% CLA mixed TG and four mice were continued on the CON diet for the last two weeks of study period and injected with either PBS (CON-PBS) or ROSI (CON-ROSI and CLA-ROSI). Values represent mean ± SE. Superscripts represent significant differences between treatments. Differences between means was calculated using one way ANOVA, values were considered significant at P<0.05.
**Insulin tolerance:**

Compared to CON-PBS group, ROSI significantly improved insulin sensitivity in both CON- and CLA-fed *ob/ob* mice and CLA-ROSI fed mice had insulin sensitivity comparable to CON-ROSI mice (AUC; Table 4.2 and Figure 4.5).

**Liver and Muscle TG:**

While ROSI treatment did not decrease hepatic lipids in CON fed mice, interestingly, there was a significant reduction in liver TG in the CLA-ROSI group compared to CON-PBS and CON-ROSI groups after two weeks of ROSI treatment (Table 4.2). There was no effect of diet or treatment on muscle TG in *ob/ob* mice (Figure B.1, p.172).
Figure 4.5 Effect of dietary CLA on insulin sensitivity: Male \( ob/ob \) mice from study 2. Insulin sensitivity was measured after two weeks on either control (CON) or CLA-supplemented (CLA) diets and injected with PBS or ROSI. CON-PBS (\( n=8, \) closed circles), CON-ROSI (\( n=4, \) open circles), CLA-ROSI (\( n=6, \) closed triangles). Significance was determined using AUC (Table 4.2).
Liver mRNA expression:

Because the combination of CLA with ROSI had significantly lower liver TG, we measured mRNA levels of genes indicative of lipid oxidation and lipid synthesis in the liver. ROSI treatment significantly increased mRNA level of liver AOX in both CON- and CLA-fed mice compared to CON-PBS mice (Figure 4.6A). There were no significant differences in the mRNA levels of peroxisome proliferator activated receptor (PPAR-α) and carnitine palmitoyl transferase (CPT-1) between the groups (data not shown). While ROSI treatment significantly increased mRNA levels of FAS in the CON fed mice, interestingly, the combination of CLA with ROSI had significantly lower levels of FAS mRNA comparable to CON-PBS mice (Figure 4.6B).

Muscle mRNA expression:

Although muscle lipid content did not differ between diet groups, we measured mRNA levels of fatty acid transporter - CD36 and AOX that have been previously shown to be up-regulated by adiponectin and have important functions in lipid metabolism (209). ROSI treatment significantly increased CD36 and AOX mRNA levels in mice fed dietary CLA (Figure 4.6C-D) compared to mice fed the CON diet.

Adipose mRNA expression:

ROSI administration significantly decreased TNF-α mRNA levels in both CON- and CLA-fed mice compared to PBS injected mice fed the CON diet (Figure 4.6E).
Figure 4.6 Effect of CLA on mRNA expression of genes involved in lipid metabolism from study 2: Male ob/ob mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either PBS or ROSI for two weeks by IP injection daily. CON-PBS (n=8), CON-ROSI (n=4), CLA-ROSI (n=6) (A) hepatic AOX mRNA; (B) hepatic FAS mRNA; (C) muscle CD36; (D) muscle AOX; (E) epididymal adipose TNF-α. Vertical bars represent mean ± SE, * P<0.05.
4.5 DISCUSSION

The effects of CLA on insulin sensitivity are controversial and vary depending on the species and level of dietary fat. Preliminary data from male and female ob/ob mice showed that feeding dietary CLA (1.5% mixed isomer) as a part of 6.5% total fat diet for four weeks resulted in a significant decrease in body weight gain and adiposity along with an increase in hepatic steatosis (Tables B.1 and B.2). The decrease in body weight measurements was significant by day seven in mice fed dietary CLA (Figure B.4, p.177). Along with decrease in weight gain, dietary CLA feeding resulted in a significant decrease in serum adiponectin (Figure B.3, p.175) and an increase in FBG levels by two weeks (Table B.3, p.178). Serum levels of resistin were not increased by dietary CLA in ob/ob mice after four weeks. Further, adipose mRNA levels of inflammatory cytokines e.g. TNF-α and IL-6 were also not significantly different between CON and CLA groups after four weeks (Table B.3, p.178).
In the present study, we show that CLA causes rapid changes in weight and adiposity in mice. The depletion in adipose tissue is accompanied by worsened insulin sensitivity and development of hepatomegaly (likely attributable, in part, to increased hepatic lipids). These results are in accordance with other studies using both mixed isomers of CLA as well as the t10c12 isomer alone (115-119). Previously, it was shown that CLA causes a time course depletion of adiponectin and leptin and is associated with the development of insulin resistance in female C57BL/6J mice (118). Here, we show for the first time that removing CLA from the diet results in reversal of depressed adipokines, insulin resistance, and hepatic steatosis.

In study 1, the level of adiponectin was significantly depleted at six days in male C57BL/6 mice supplemented with dietary CLA. These results are in accordance with a study conducted by Poirier et al (118). However, in contrast to their study, in the present study, using comparable levels of dietary CLA, leptin levels were not significantly lower than baseline. These differences may be due to differences in gender in the two studies. Associated with the depletion of adiponectin, there was a significant decrease in phosphorylation of key mediators of glucose and lipid metabolism pathways such as AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in the livers of C57BL/6 mice and levels of these phospho-proteins were restored by removing CLA from the diet (Figure B.2, p.173). The observed decrease in phosphorylation state of AMPK and ACC may be indicative of decreased lipid oxidation in the liver and impaired insulin responsiveness (45; 216-218). Switching mice to the diet without CLA resulted in a significant improvement in serum adiponectin levels as early as seven days. Of interest here, is that while insulin resistance and hepatic TG were significantly attenuated when
CLA was removed from the diet, adiponectin levels were only partially restored suggesting that the presence of leptin may compensate for insufficient adiponectin levels and the overall adipokine status may influence the response to dietary CLA in different animal models. The concomitant repletion of adipokines and reversal of insulin resistance by removing CLA from the diets is novel and further supports a strong association between the dysregulation of adipokine synthesis by dietary CLA and worsening of insulin sensitivity in mice.

Previously, it was demonstrated that leptin infusion into C57BL/6 mice reverses insulin resistance and hepatic steatosis when adipose stores are not completely ablated (116). However, leptin has no effect when infused into A-ZIP/F-1 fatless mice (219). These data suggest that leptin insufficiency alone is not the principal cause for lipodystrophy-associated insulin resistance caused by CLA (219). The effects of CLA on insulin resistance and hepatic steatosis have been previously demonstrated in ob/ob mice by Roche et al. (115). Preliminary findings from our study are in agreement with the study conducted by Roche et al. In addition, we measured serum adiponectin levels over time in female ob/ob mice fed CLA and found that levels were significantly and maximally depressed by two weeks (Figure B.3, p.175). Further, mice developed significant hepatic steatosis and insulin resistance along with the depletion of adiponectin (Table B.1, p.174).
Because ob/ob mice lack functional leptin, these data in conjunction with our data from study 1 suggest that the worsening of insulin resistance caused by dietary CLA may be more strongly associated with depletion of adiponectin or overall adipokine status than leptin alone. To further examine the role of adiponectin in CLA induced insulin resistance, we injected leptin deficient male ob/ob mice with an adiponectin enhancer; ROSI for two weeks.

ROSI treatment significantly increased serum concentrations of adiponectin in mice fed either the CON or CLA diet compared to CON-PBS treated mice. These effects of ROSI on serum adiponectin are consistent with other studies (209; 220; 221). Because higher circulating levels of serum resistin are often associated with insulin resistance and diabetes in rodents (222), we measured serum levels of resistin. Preliminary data from male ob/ob mice fed dietary CLA for four weeks did not have significantly increased resistin levels compared to CON-PBS mice (Table B.3, p.178). Further, ROSI treatment did not have an effect on serum resistin levels in ob/ob mice fed dietary CLA. The lack of effect of ROSI on serum resistin levels in CLA fed mice further demonstrates that CLA mediated insulin resistance is related more to the depletion of adiponectin. The increase in serum adiponectin in the CLA-fed mice was accompanied by significantly lower levels of fasting glucose, insulin, insulin sensitivity, serum TG, and NEFA similar to CON-ROSI treated mice. Co-treatment of CLA with ROSI for two weeks prevented lipodystrophy and associated increases in serum levels of glucose and insulin that are usually associated with supplementation of dietary CLA in mice (116-119). Additionally, ROSI treatment also prevented significant body weight loss which is often reported with treatment of CLA in mice. This may be attributed to maintenance of adipose mass as
ROSI, a PPAR-γ agonist, often increases adipose mass (213; 214). These data suggest that when adipose mass and adiponectin levels are maintained, the lipodystrophic effects associated with dietary CLA supplementation are attenuated.

In the present study, increased adiponectin levels were associated with higher levels of hepatic AOX mRNA in CON- and CLA-fed mice but only CLA-ROSI group had significantly lower hepatic FAS mRNA levels and hepatic TG. To our knowledge, the interactive effect of the combination of CLA and ROSI on hepatic TG is novel and suggests a complementary effect of these two agents for restoring normal lipid levels in the liver. Further, there were significant increases in the levels of the lipid transporter CD36 and lipid oxidative enzyme, AOX in the muscle of the CLA-ROSI group. Administration of adiponectin has similar effects on CD36 and AOX mRNA in mice fed high fat diets (209), suggesting that at least a part of effects of ROSI are mediated through adiponectin. Although it is unclear why the combination of CLA with ROSI has additional effects in the liver and muscle, it may be due to increased adiponectin levels with ROSI administration. It is possible that in the presence of adequate adiponectin, CLA increases lipid utilization and decreases lipid accumulation in tissues similar to observations from rat studies (111; 112; 223).
It has been shown previously that supplementation with dietary CLA significantly decreases adipokines and induces hyperinsulinaemia in female C57BL/6J mice by six days (118). In the present study, ROSI was administered for two weeks. In this short term treatment, ROSI prevented CLA induced insulin resistance by maintaining adequate adiponectin levels in ob/ob mice. While we cannot speculate the effects of ROSI if treatment had been prolonged in this model, these results are in accordance with a recent study conducted in C57BL/6 mice (224) that were administered the combination of CLA-ROSI for six weeks.

Because other adipokines secreted from the adipose tissue such as TNF-α, IL-6 and resistin are known to modulate insulin sensitivity in addition to leptin and adiponectin, the overall adipokine status and not any particular adipokine alone may influence CLA’s effects on insulin resistance and hepatic steatosis. It was recently shown that short term t10c12-CLA administration induced adipose IL-6 and TNF-α mRNA without affecting serum levels (120). In contrast to these findings, preliminary data from male ob/ob mice fed mixed isomers of CLA for four weeks did not have significantly higher mRNA levels of TNF-α and IL-6 compared to CON mice (Table B.3, p.178). Thus, while in the present study, adiponectin alone seems to be more important than other adipokines in insulin resistance mediated by CLA, future studies using adiponectin deficient mice are necessary to make conclusions regarding the relative importance of this adipokine. ROSI treatment significantly decreased adipose tissue TNF-α mRNA levels in both CON- as well as CLA- fed mice compared to CON-PBS treated mice. These data are in accordance with previous reports (225; 226). Because ROSI is a thiazolidinedione known to improve insulin sensitivity possibly through multiple
pathways, the increased synthesis of adiponectin is not the only possible explanation for our findings. In fact, ROSI may have additional effects in the adipose tissue and may decrease insulin resistance mediated by CLA by directly or indirectly modulating inflammatory cytokines such as TNF-α and IL-6.

In conclusion, we show that removing CLA from the diet of mice reverses insulin resistance and restores adiponectin establishing a link between depletion of this adipokine and development of hepatic steatosis and insulin resistance by dietary CLA in mice. We further show that in the absence of significant changes in leptin levels (male C57BL/6 mice fed dietary CLA) or in mice lacking functional leptin (male ob/ob mice), adiponectin depletion alone results in worsened insulin sensitivity by CLA. In addition, restoration of adiponectin (by either removal of CLA from diet or administering ROSI) is sufficient for reversal of these effects. Thus, in the present study, we show that adiponectin is an important factor responsible for insulin resistance caused by CLA provided as mixed isomer oil at 1.5 wt% of the diet. We based our dose (1.5% CLA equaling approximately 0.6% t10c12-CLA) on studies by others showing this dose to be effective for adipose suppression in mice. If expressed per kg body weight for a human of ~55 kg would equal ~68 g CLA oil per day. With this estimate, it seems unlikely a feasible choice for humans to use and achieve such a rapid loss of body fat. However, there are other factors to consider between species (mice vs. humans) including rapidity of metabolism of CLA isomers as well as rapidity of adipose tissue metabolism and specific to this design adipose catabolism. We consider these pre-clinical studies to be important for improving our understanding of mechanisms of anti-adipose effects of CLA.
4.6 ACKNOWLEDGMENTS

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CHAPTER 5

Citrus fruit flavonoid naringenin suppresses hepatic glucose output from cultured hepatocytes

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Abbreviations: AMPK, AMP-activated protein kinase; Dex, dexamethasone; G-6pase, glucose-6-phosphatase; HGP, hepatic glucose production; PI3kinase, Phosphatidylinositol 3-kinase; PEPCK, phosphoenolpyruvate carboxy kinase.
5.1 ABSTRACT

Hepatic gluconeogenesis is the major source of fasting hyperglycemia in diabetes. Here, we investigated the role of the citrus fruit flavonoid naringenin, found predominantly in grapefruits, in the attenuation of glucose production from Fao hepatoma cells. Incubating Fao cells with naringenin but not the glucoside naringin, in gluconeogenic medium, significantly suppressed glucose production from cells in a dose dependent manner. In contrast to insulin, naringenin suppressed glucose production in a PI3kinase independent manner as incubation with the PI3kinase inhibitor Ly294002 did not attenuate the ability of naringenin to suppress glucose output from Fao cells. Furthermore, naringenin did not increase p-Akt compared to cells incubated with Veh alone, suggesting that the glucose suppressive effects of naringenin may be independent of the PI3kinase signaling pathway. Metformin was used as a positive control in the present study and both naringenin and metformin significantly decreased cellular ATP levels along with increases in p-AMP activated protein kinase. Together, these results suggest that the aglycone naringenin has a role in the attenuation of hyperglycemia, similar to the drug metformin.
5.2 INTRODUCTION

Increased hepatic glucose production (HGP) contributed by both gluconeogenesis and glycogenolysis is the major cause for elevated fasting blood glucose levels in both type I and type II diabetes (7). Gluconeogenesis alone contributes to approximately 50-60% of the endogenous glucose production (227; 228) and is regulated by rate limiting enzymes such as phosphoenolpyruvate carboxy kinase (PEPCK) and glucose-6-phosphatase (G-6-Pase). The rate of transcription of these key enzymes is up-regulated by several hormones including glucocorticoids and glucagon (mediated by its second messenger cAMP). Insulin is the main suppressor of gluconeogenesis and impaired insulin secretion or action together with increases in glucagon result in elevated hepatic glucose production (7; 11; 228; 229).

In addition to drugs, a great deal of attention has been given to naturally occurring compounds of plant and animal origin for the attenuation of hyperglycemia. For instance, polyphenolic compounds of plant origin have been used traditionally to treat several chronic conditions [reviewed in (230)]. One such naturally occurring compound is the flavanone naringenin which has anti-atherogenic and plasma lipid lowering effects in rodents (164-166; 177). Furthermore, a single i.p. injection of 7-O-b glucoside form of naringenin reduced blood glucose, TG and cholesterol in diabetic rats (174). Naringenin exists as both glycoside-naringin and aglycone-naringenin and is mainly derived from citrus fruits, especially grapefruit, which contains approximately 78mg naringenin/100g
grapefruit (173). Very few studies have assessed its role in diabetes and to our knowledge the effect and mechanism of action of naringenin, the aglycone form, in lowering blood glucose is still unknown.

Recently, the glucose lowering effects of the glucoside form naringin were demonstrated in db/db mice, a murine model for type 2 diabetes which lack functional leptin receptors. Feeding naringin at 0.2 g/kg diet to db/db mice significantly attenuated hyperglycemia in part by suppression of gluconeogenesis along with an increase in glycogen synthesis (171). In contrast to this novel finding, no studies have reported an effect of the aglycone form of naringin, naringenin on hyperglycemia. The majority of food flavonoids are bound to glycosides and in general, thought to be less bio-available than the aglycone forms (157; 158; 231). Further, a recent study using labeled naringenin reported that it was the aglycone form that accumulated extensively in tissues 18hr post-gavage in rats (232). Thus, the objectives of the present study were to determine the glucose lowering effects of the aglycone naringenin in vitro and understand the molecular mechanisms behind the glucose lowering effects of this citrus fruit flavonoid.
5.3 MATERIALS AND METHODS

Materials:

Rat hepatoma cells (H4IIE) cells (Fao cells) were a gift from R.C. Kahn (Joslin Diabetes Center, Harvard, MA). Naringenin was purchased from Sigma (St. Louis, MO). Cell culture medium, fetal bovine serum (FBS) and Trozol were purchased from Invitrogen-Gibco (Carlsbad, CA). Naringenin, naringin, cAMP analogue, 8-Ctp cAMP, synthetic glucocorticoid, dexamethasone (dex), sodium lactate, sodium pyruvate and insulin from porcine pancreas were purchased from Sigma (St. Louis, MO). Glucose determination kit was purchased from Sigma Inc (St. Louis, MO). Naringenin and Ly294002 (Cell Signaling Technology, Beverly, MA) were solubilized in dimethyl sulfoxide (DMSO) purchased from Sigma (St. Louis, MO). PEPCK antibody was a gift from Dr. Colleen Croniger (Case Western Reserve University, Cleveland, OH).

Glucose output experiments:

Glucose output experiments were performed using a modified protocol from Kahn et al. (229). Fao cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and plated on 6 well dishes at 1.6x 10^6 cells per well. Experiments were performed once cells reached confluence. At confluence, medium was changed to 0.1% FBS DMEM and incubated overnight to equilibrate cells. Next morning, cells were washed and incubated in gluconeogenic medium (GNG) - glucose free, phenol red free DMEM containing 20mM Na lactate and 2mM Na pyruvate as
indicated. Cells were stimulated with 8-Ct-p-cAMP (100uM) plus dex (500nM) to induce gluconeogenesis and treated with naringenin and naringin as indicated. Medium was collected at the end of the incubation period and analyzed for glucose secreted into the medium using glucose oxidase kit. Cells were washed 2x with ice cold PBS, harvested and lysed in 10x Tris (w/v) buffer containing 20mM trizma base, 1% tritio-n-X100, 50mM NaCl, 250mM sucrose, 50mM NaF, 5mM Na4P2O7.10H2O and protease inhibitors. Glucose production was normalized to protein content per sample. In addition to naringenin, insulin and metformin (dimethylbiguanide) were used in all experiments as positive controls. Ly294002 a specific PI3kinase inhibitor was used as indicated. All experiments were performed at least three times and representative data are shown.

**Real time RT PCR:**

Fao cells were incubated in GNG medium for 6hr with naringenin, insulin and metformin and cells were harvested in Trizol reagent and RNA was isolated using the manufacturer’s protocol. Extracted RNA was diluted in RNase-free water and quantified by spectrophotometry. RNA integrity was assessed by electrophoresis using agarose gel and ethidium bromide staining. The first transcripts were reverse transcribed using reverse transcriptase (Applied Biosystems, Foster City, CA) and cDNA was amplified using real-time PCR with FAM labeled TaqMan gene expression assays (Applied Biosystems, Foster City, CA). In short, 10 ng of the reverse transcription reaction was amplified in a total reaction volume of 25µl using pre-designed and validated primers for liver PEPCK and G-6Pase using universal cycling conditions. Target gene expression was normalized to Vic labeled18s, which was used as an endogenous control and amplified in the same reaction as the target gene.
**Western Blotting:**

Western blot analysis was performed for detecting proteins that modulate glucose metabolism. Cells were incubated in GNG medium containing cAMP + Dex with insulin, naringenin and metformin for as indicated. Cells were lysed in 10x Tris (w/v) buffer containing 20mM trizma base, 1% trition-X100, 50mM NaCl, 250mM sucrose, 50mM NaF, 5mM Na₄P₂O₇·10H₂O and protease inhibitors for 30min on ice. Samples were frozen and thawed 2-3 times to achieve complete lysis and centrifuged at 12000 x g for 10 min. The supernatant was separated as the total soluble fraction. Protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes (BioRad, Hercules, CA). Membranes were blocked overnight at 4°C with primary antibodies to detect p-Akt (1:1000), p-AMPK (1:1000) and PEPCK (1:5000) followed by incubation with HRP linked secondary antibody (1:2500). Polyclonal antibodies to p-Akt (Ser473 and Thr308) and p-AMPK (Thr 172) were purchased from Cell Signaling Technology (Beverly, MA). Total Akt, (1:1000) and AMPK (1:1000) and β-actin (1:1000) Ab were used to normalize values. Proteins were detected using super signal chemiluminescence system (Pierce Biochemicals, Rockford, IL). Blots were quantified using densitometry (Kodak imager system).

**Cell viability:**

Fao cells were plated at 1.6x 10⁶ cells per well on 6 well plates in low glucose DMEM and at confluence; cells were incubated with treatments in GNG medium for 6 hrs. At the end of the incubation period, cells were trypsinized and cell viability was determined using trypan-blue exclusion method.
**ATP determination assay:**

Cellular ATP content was determined using cell titer-Glo luminescent cell viability assay (Promega, Madison, WI) in a reaction catalyzed by recombinant firefly luciferase. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg$^{2+}$, ATP and oxygen generating luminescence proportional to the amount of ATP present in the cells. Fao cells were cultured as described previously and plated in 96 well plates in low glucose DMEM. Cells were stimulated with cAMP +Dex and treated with DMSO (Veh), naringenin (0-200uM), metformin (1mM) in glucose free, phenol red free DMEM containing gluconeogenic precursors (Na Lactate and Na Pyruvate) for 3 and 6hrs. Relative luminescence was normalized to total protein content/sample.

**Statistical analysis:**

All data are presented as mean ± SE. Data were analyzed using MINITAB (version 14, PA). Data were analyzed by one-way ANOVA. Post-hoc analysis was performed using Tukey’s test. Differences were considered significant at P < 0.05.
5.4 RESULTS

*Naringenin inhibits HGP in a dose dependent manner*

Glucose production from Fao cells was measured as described in the methods section. Glucose production was significantly increased in cells stimulated with 100µM 8-Ctp-cAMP plus 500nM dex with or without Dmsö (Veh) compared to un-stimulated cells (C) and treatment with 100nM insulin (IN) significantly decreased glucose production in stimulated cells. Treatment with naringenin significantly decreased glucose production in a dose dependent manner (P<0.05) compared to Veh treatment (Figure 5.1A). The glucose suppressive effect of naringenin was greatest with the 100 µM dose. Metformin (1mM), a known inhibitor of gluconeogenesis also significantly decreased glucose production. There were no additive effects of naringenin and insulin on the suppression of glucose production in Fao cells (Figure C.1, p.179).

In order to determine the glucose suppressive effects of naringenin over time, Fao cells were incubated in GNG medium as indicated (Figure 5.1B). Naringenin (N; 100 µM), metformin (M; 1mM) and insulin (IN; 100nM) significantly decreased glucose output as early as 2hrs in stimulated cells compared to Veh treated cells.. The glucose lowering effect of both metformin and naringenin persisted up to 24 hr. Glucose levels were undetectable in the medium until 3hr with 100µM naringenin and although glucose production was significantly increased at 6hr, it was significantly lower compared to Veh treated cells (Figure 5.1B).
The glucose suppressive effect of naringenin was compared with its glucoside naringin in Fao cells incubated in GNG medium for 6hr. Insulin (IN; 100nM) was used as a positive control. While naringenin (N; 100µM) significantly suppressed glucose production from Fao cells compared to Veh treated cells, its glucoside naringin (NAN; 100µM) had no effect on inhibition of glucose output (Figure 5.1C).
Figure 5.1 Effect of naringenin on HGP. Naringenin decreases hepatic glucose production in Fao hepatoma cells stimulated with cAMP + Dex. (A) Naringenin (N; 6-100µM), insulin (IN; 100nM) and metformin (M; 1mM) decrease HGP compared to Veh (DMSO) treated cells after 6hr of incubation in GNG medium. Un-stimulated cells (C). (B) Effect of Veh (DMSO), naringenin (100µM), insulin (100nM) and metformin (1mM) on HGP over time. Fao cells were incubated as indicated in GNG medium stimulated with cAMP + dex. Superscripts represent significant differences between treatments within time points. (C) Naringenin (N) but not glucoside naringin (NAN) significantly suppress HGP from Fao cells after 6hr in GNG medium. Values represent mean + SEM. Superscripts represent significant differences between treatments, P< 0.05.
Inhibition of HGP by naringenin is independent of PI3kinase signaling

Insulin regulates HGP in a PI3kinase dependent manner. To test whether the suppressive effect of naringenin, like insulin, was dependent on activation of PI3Kinase, cells were treated with a specific PI3kinase inhibitor, Ly294002 (30µM) at time 0, and at time 30min, Veh, naringenin (N; 100µM), insulin (IN; 100nM) and metformin (M; 1mM) were added to the GNG medium and incubated for 6hr. While Ly294002 completely inhibited insulin’s and metformin’s suppressive effects on glucose output, there was no effect of Ly294002 on the ability of naringenin to suppress glucose production (Figure 5.2A-B).

Activated Akt/protein kinase B (PKB), phosphorylated by insulin, is a downstream signaling molecule in the PI3kinase pathway and is a key regulator of glucose metabolism. To determine if naringenin, like insulin mediates its glucose suppressive effects via this molecule, phosphorylation of Akt was determined using western blotting. Fao cells were incubated in GNG medium with insulin (IN; 100nM), naringenin (N; 100µM) and metformin (M; 1mM). Akt activation was determined by measuring phosphorylation at Ser473 and Thr308 residues after 6hr. Data were normalized to total Akt. While insulin increased phosphorylation of the Ser473 residue on Akt significantly compared to Veh treated cells, naringenin did not increase Akt phosphorylation at this residue. Thr308 phosphorylation was not altered significantly by any treatments (Figure 5.2C). In addition, p-Akt was measured after 3 and 9 hr of incubation with insulin and naringenin. Insulin but not naringenin increased Akt phosphorylation at both time points (Figure C.2, p.180).
Figure 5.2 Naringenin decreases HGP independently of PI3kinase signaling in Fao cells. (A) Ly29002 (30µM; hashed bars) significantly attenuates the suppressive effect of insulin and metformin (M; 1mM) but not naringenin on HGP. At time 0, Ly29002 (30µM) was added, and at time 30min, insulin (IN;100nM), metformin (M;1mM) and naringenin (N;100µM) were added in GNG medium. (B) Ly29002 does not attenuate the effect of naringenin (100µM) on cAMP + Dex induced HGP. At time 0, Ly29002 (10, 30, 50µM) was added and at time 30min, naringenin (N; 100µM) was added in GNG medium for 6hr. Values represent mean + SEM. Superscripts represent significant differences between treatments, P<0.05. (C) Insulin (IN; 100nM) but not naringenin (N; 100µM) or metformin (M; 1mM) activate Akt in Fao cells. Insulin increased phosphorylation of the Ser 473 residue (hashed bars) compared to Veh treated cells. Phosphorylation of the Thr 308 (solid bars) residue was unaffected by any treatment. Treatments were in GNG medium in the presence of cAMP + dex for 6hr. Lanes: 1,2 Veh; 3,4 naringenin; 5,6 metformin; 7,8 insulin. Values represent mean + SEM. Superscripts represent significant differences between treatments for the Ser 437 residue, P<0.05.
A

Ly 29002 (30μM)

C_Veh IN M N + IN + M + N

Glucose (ng/sample protein)

0 2 4 6

Ly 29002 (30μM)

B

cAMP + dex

Veh N N+Ly10 μM N+Ly30 μM N+Ly50 μM

Glucose (ng/sample protein)

0 1 2 3 4 5 6

P-AKT Ser 473

P-AKT Thr 308

AKT

Arbitrary units (p-Akt/total Akt)

0.0 0.5 1.0 1.5 2.0 2.5

Veh IN N M
Measurements of PEPCK mRNA and protein

Glucose metabolism is regulated by rate limiting enzymes such as PEPCK. In order to determine the mechanism of action of naringenin in suppressing glucose production from Fao cells, we measured mRNA and protein levels of PEPCK. While treatment with cAMP + dex significantly increased mRNA and protein levels of PEPCK compared to un-stimulated cells (C), insulin (IN; 100nM) significantly suppressed PEPCK transcript and protein levels in stimulated cells. In contrast, naringenin (N; 100µM) and metformin (M; 1mM) did not alter either mRNA or protein expression of PEPCK (Figure 5.3A-B).
Figure 5.3 Naringenin does not inhibit the rate limiting enzyme PEPCK expression in Fao cells. (A) Treatment with naringenin (N; 25, 50 or 100μM) or metformin (M; 1mM) does not decrease cAMP + dex induced PEPCK transcript levels in cells treated for 4hr in GNG medium. Insulin (IN; 100nM), was used as positive control. (B) Protein levels of PEPCK measured by western blotting were unchanged by naringenin (N; 25, 50 or 100μM) and metformin (M;1mM) but levels were significantly reduced by insulin (IN; 100nM) in cells stimulated with cAMP + dex after 9hr of incubation in GNG medium. Lanes: 1,2 DMSO; 3,4 naringenin (100μM); 5,6 insulin;7,8 metformin, 9 C. Values represent mean ± SEM. Superscripts represent significant differences between treatments, P<0.05.
Naringenin does not affect substrate utilization

Because lactate uptake into the mitochondria is the rate limiting step for its conversion to oxaloacetate (OAA) and subsequently, its export into the cytosol for conversion to glucose, we tested the possibility that naringenin exerted its effects on inhibition of HGP by decreasing lactate uptake into mitochondria. Glucose production from Fao cells was measured using 20mM Na lactate, oxaloacetate (OAA) or glycerol and 2mM Na pyruvate. Because OAA and glycerol can bypass the uptake into mitochondria, we hypothesized that the ability of naringenin to suppress glucose output is blunted with these substrates. Naringenin significantly suppressed glucose production compared to Veh treated cells regardless of the substrate used (Figure 5.4A-C). Similarly, neither insulin nor metformin’s ability to suppress glucose production was altered by OAA or glycerol in Fao cells.
Figure 5.4 Effect of naringenin on lactate uptake. Naringenin (N; 100µM) decreases cAMP + dex induced HGP in Fao cells from (A) 20mM Na lactate + 2mM Na pyruvate; (B) 20mM OAA + 2mM Na pyruvate and (C) 20mM glycerol + 2mM Na pyruvate. Values represent mean + SEM. ** represent significant differences between treatments at each time point, P<0.05.
**Cellular ATP determination and cell viability:**

Recent evidence suggests that metformin inhibits gluconeogenesis by decreasing the mitochondrial ATP production via inhibition of complex I of the respiratory chain (233). In order to test the possibility that naringenin exerts its effects on glucose production by a similar mechanism, cellular ATP concentrations were measured using a luciferase based assay. Compared to Veh treated cells, naringenin (N; 50 and 100µM) and metformin (M; 1mM) significantly decreased ATP concentrations after 3 and 6 hrs of incubation ([Figure 5.5 A-B](#)). These changes were observed in the absence of changes in viability and protein levels ([Figure C.4, p.182](#)).
Figure 5.5 Effect of naringenin on cellular ATP concentrations. Naringenin (25, 50 and 100µM) and metformin (M; 1mM) significantly decreased cellular ATP concentrations compared to Veh treated cells after 3 (A) and 6hr (B). Fao cells were incubated in GNG medium and stimulated with cAMP + dex. Cellular ATP levels were measured using a luciferase based assay. Values represent mean ± SEM. Superscripts represent significant differences between treatments at each time point, P<0.05.
Measurement of p-AMPK

AMP-activated protein kinase (AMPK) has been identified as a target capable of mediating metformin’s effects on glucose output suppression (234). A low cellular ratio of ADP/ATP is linked to the activation of AMPK by increasing AMPK phosphorylation. Therefore, we measured p-AMPK levels in Fao cells incubated with naringenin (N; 100µM) and metformin (M; 1mM) in low glucose (5.5mM) DMEM by immunoblotting. 5-aminoimidazole-4-carboxamidine-1-β-D-ribofuranoside (AICAR) was used as a positive control. Both metformin and naringenin increased p-AMPK as early as 1hr in Fao cells compared to Veh treatment and effects lasted up to 7hr (Figure 5.6 A-B).
Figure 5.6 Effect of naringenin on AMPK. Naringenin (N; 100µM) and metformin (M; 1mM) increase p-AMPK levels compared to Veh treated cells. Cells were incubated in low glucose DMEM for 1hr (A) and 7hr (B) and p-AMPK levels were determined by immunoblotting. AICAR (A; 500µM) was used as a positive control. (C) Lanes: 1,2 Veh; 3,4 naringenin; 5,6 AICAR; 7,8 metformin. (D) Lanes: 1,2 Veh; 3,4 naringenin; 5,6 metformin; 7,8 AICAR. Values represent mean ± SEM. Superscripts represent significant differences between treatments at each time point, P<0.05.
5.5 DISCUSSION

In the present study, we show for the first time that the aglycone naringenin is a potent suppressor of hepatic glucose production most likely involving similar mechanisms as the widely used drug metformin for the attenuation of hyperglycemia. Fao cells, derived from the H4IIE hepatoma line possess a complete compliment of gluconeogenic enzymes and can survive in glucose free medium (229). Therefore, in the present study, we chose this in vitro model to determine the effects of naringenin on HGP.

Attenuation of hyperglycemia is a major treatment goal for the management of diabetes. Diabetes is characterized by increases in fasting glucose levels, contributed mainly by increased HGP (7; 9). Therefore, agents that suppress HGP can be beneficial for the management of this disease. In the present study, naringenin significantly suppresses HGP stimulated by the cAMP analogue, 8-ctp-cAMP and the synthetic glucocorticoid dex in Fao cells. It has been previously demonstrated that Fao cells maintained in low glucose medium (5.5mM) lack substantial amounts of glycogen (11) and thus, it can be concluded that the glucose detected in the medium was essentially contributed by de novo synthesis from non-hexose precursors e.g. lactate and pyruvate.

In the present study, naringenin robustly inhibited glucose production from Fao cells and the suppression of HGP was similar to that of insulin and metformin. However, the magnitude of suppression of glucose production by naringenin was significantly greater than metformin up to 6hr after which time, suggesting that naringenin may alter
early events in the activation of gluconeogenic pathway. Furthermore, naringenin suppressed glucose output at lower doses (25uM-100uM) than metformin which is effective only at much higher doses (1-2mM) for immediate effects (234). The rate of gluconeogenesis is controlled by key enzymes e.g. PEPCK and G-6Pase that are inhibited transcriptionally by insulin in a PI3kinase dependent manner (7; 74; 229). In the present study, neither naringenin nor metformin had any significant suppressive effects on the mRNA or protein expression of PEPCK. This is in contrast to a recent study published by Jung et al., in db/db mice (171) where it was demonstrated that the glucoside naringin significantly attenuated hyperglycemia by suppressing PEPCK and G-6Pase activity. Furthermore, they demonstrated significant increases in the glycolytic enzyme, glucokinase activity along with increased hepatic glycogen stores in naringin fed mice compared to control animals. In contrast to these in vivo findings, in the present study, naringenin but not the glucoside naringin significantly suppressed HGP when used at comparable doses. Naringin is the glucoside form of the flavonoid and undergoes cleavage of the glucose moiety bacteria in the gut before it is absorbed (178). Therefore, it is possible that the effects of naringin observed by Jung et al., (172) were in fact, in fact due to naringenin which is possibly the active form.

In the present study we did not observe significant effects of insulin, cAMP or naringenin on G-6pase expression (Figure C.3, p.181). These results may be explained by a previous study by Argaud et al., (235), who reported that Fao cells express very low levels of both glucokinase and G-6Pase as G-6Pase expression is dependent on the expression of glucokinase expression. Metformin has been shown to decrease G-6pase activity along with increased glycogen stores with minimal effects on PEPCK gene
expression in rats (236; 237). Similar lack of effect of metformin and naringenin on PEPCK mRNA expression was observed in the present study using Fao cells and it is possible that like metformin, naringenin may exert its glucose lowering effects by inhibiting G-6Pase expression and needs to be examined in future studies using in vivo models and primary hepatocytes. Another possible mechanism by which naringenin exerts its biological activity may be via increased glycolytic flux. Like G-6pase, the expression of pyruvate kinase has been reported to be very low in Fao hepatoma cells. Therefore, future studies using primary cell lines or in vivo models may help to clarify the mechanism of action of naringenin.

In order to test whether activation of PI3kinase is required for the glucose suppressive effects of naringenin, we used a specific PI3kinase inhibitor Ly294002 with naringenin. While Ly294002 significantly attenuated both insulin’s and metformin’s ability to suppress HGP, inhibition of PI3kinase activity had no effect on naringenin’s ability to suppress HGP. Furthermore, in the present study, naringenin did not significantly increase phosphorylation of Akt at the Ser473 residue. Because Akt/PKB activation by PI3kinase is an important signaling event responsible for the metabolic actions of insulin, these data suggest that naringenin, unlike insulin, mediates its effects on HGP in a non-PI3kinase dependent manner. This is in contrast to a study in HepG2 cells where naringenin’s ability to decrease Apo B secretion was dependent on PI3kinase activation (165). These differences may be species or tissue specific as naringenin has been shown to inhibit PI3kinase activity in 3T3L1 adipocytes (238).

Metformin (dimethylbiguanide) is a widely used compound for the attenuation of hyperglycemia. Postulated mechanisms of action to suppress gluconeogenesis include
inhibition of uptake of gluconeogenic precursor lactate, inhibition of rate limiting enzyme activities (mainly G-6Pase) and increased flux through pyruvate kinase secondary to decreased cellular ATP content (233; 234; 237; 239; 240). In the present study, we explored some of these possible mechanisms with naringenin. To test the possibility that naringenin decreased HGP by blocking mitochondrial lactate uptake, we used OAA and glycerol, as these substrates are intermediates in the gluconeogenic pathway that are mainly present in the cytosol and do not require uptake into mitochondria. In the present study, naringenin’s ability to suppress HGP was not altered when OAA and glycerol were used as substrates compared to comparable concentrations of lactate. These data suggest that naringenin’s effect on HGP suppression is not dependent on substrate uptake in Fao cells. Further, in contrast with studies using isolated rat hepatocytes showing decreased lactate uptake with metformin (241), in Fao cells we did not see differences in glucose output when lactate, OAA or glycerol were used.

There is substantial evidence suggesting that metformin suppresses HGP by increasing AMPK activation (234; 237). In the present study, incubating Fao cells with naringenin and metformin significantly increased p-AMPK compared to Veh treated cells. Recent evidence suggests that metformin is not a direct AMPK activator but exerts its anti-diabetic effects by inhibition of the mitochondrial respiratory chain complex I (233; 239). Inhibition of the respiratory chain complex results in decreased cellular ATP synthesis thereby increasing cytosolic ADP: ATP ratio and consequently, an increase in AMPK activation, which in turn is associated with decreased gluconeogenesis. Additionally, an increase in ADP: ATP ratio also inhibits gluconeogenesis by inhibiting pyruvate carboxylase and increasing the pyruvate kinase flux (237; 240). In the present
study, we show a similar effect of both metformin and naringenin on decreasing cellular ATP concentrations. In addition to decreases in cellular ATP content, Fao cells treated with naringenin and metformin had lower (but not significant) protein levels of cytochrome C compared to Veh treatment. While it is difficult to measure changes in free ATP/ADP ratio directly, we show here, a possible similarity between the mechanisms of actions of naringenin and metformin. In the present study, naringenin was used at doses comparable to previously reported concentrations by Borradaile et al., in HepG2 cells (165). At these concentrations, naringenin did not have any toxic effects on Fao cells as determined by trypan-blue exclusion. However, the possibility of naringenin having effects on cell cycle cannot be ruled out may need further consideration.

In conclusion, we have identified the aglycone naringenin to be the active form and a very potent suppressor of glucose output using an in vitro cell culture based model and the mechanism of action of the aglycone naringenin appears to be similar to the commonly used drug metformin. Future work using primary hepatocytes and in vivo studies will help to clarify the mechanisms and develop therapeutic targets for the effective management of diabetes and associated dyslipidemia using this potent suppressor of hepatic glucose production.
5.6 ACKNOWLEDGMENTS

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CHAPTER 6

EPILOGUE

The present study focused on understanding the mechanisms by which two unrelated bioactive compounds, CLA and naringenin, influence hepatic lipid and glucose metabolism.

In the first study, we evaluated the effects of CLA on hepatic lipid accumulation in adult Wistar rats. CLA decreased hepatic TG levels despite no changes in body weight and epididymal or peri-renal adipose tissue. Therefore, it appears that in Wistar rats, which are induced to be obese by diet (and not genetic manipulation), the effect of CLA on lowering hepatic triglycerides was not likely a result of an overall improvement in adiposity. Furthermore, the effects of CLA in adult Wistar rats occur when adipokines (e.g. leptin and adiponectin) are maintained suggesting that in the presence of sufficient adipose and/or adipokines, CLA attenuated hepatic steatosis by modulating hepatic genes involved in lipogenesis and lipid oxidation.

In the second study, we evaluated the importance of adipokines in CLA mediated insulin resistance in mice. Removing CLA from the diet of C57BL/6 mice reversed insulin resistance and restored adiponectin levels establishing a link between depletion of this adipokine and development of hepatic steatosis and insulin resistance by dietary CLA in mice. We further showed that in the absence of significant changes in leptin levels
(male C57BL/6 mice fed dietary CLA) or in mice lacking functional leptin (male *ob/ob* mice), adiponectin depletion alone resulted in worsened insulin sensitivity by CLA. In addition, restoration of adiponectin (by either removal of CLA from diet or administering ROSI) was sufficient for reversal of these effects. Thus, in the present study, we show that adiponectin is an important factor responsible for insulin resistance caused by CLA.

While these studies helped to clarify the role of adiponectin in CLA mediated insulin resistance, it still remains unclear as to why CLA mediates such opposing effects in rats and mice. It is possible that the different rate of lipid metabolism between species contributes to these differences as the magnitude of adipose tissue loss is much greater in mice than rats with dietary CLA supplementation. This may lead to the accumulation of lipids in the liver and muscle making them insulin resistant. Future studies comparing the effects of CLA in rats vs. mice to identify specific genes that may be responsible for the species specific effects are warranted.

There is a considerable lack of understanding regarding the effects of dietary CLA in humans. Randomized, placebo controlled trials in people with type 2 diabetes may help to clarify the role of CLA in humans. We based our dose (1.5% CLA equaling approximately 0.6% t10c12-CLA) on studies by others showing this dose to be effective for adipose suppression in mice. If expressed per kg body weight for a human of ~ 55 kg would equal ~ 68 g CLA oil per day. With this estimate, it seems unlikely a feasible choice for humans to use and achieve such a rapid loss of body fat. Therefore, the dose and duration of human studies may need consideration to observe any significant findings.
TZDs have been shown to increase weight gain in humans and mice. While in the present study, CLA did not decrease weight gain when supplemented with ROSI, potential beneficial effects may be observed when the length of administration is prolonged. In addition, the combination of CLA with ROSI had improved hepatic TG compared to CON mice with ROSI treatment suggesting that in the presence of adequate adiponectin levels, CLA may be beneficial for lowering hepatic steatosis.

In the second part of this dissertation, we evaluated the effects of the citrus fruit flavonoid naringenin on lowering hepatic glucose production using Fao hepatoma cells in culture. We identified the aglycone naringenin to be the active compound in lowering HGP from cells in a manner similar to the commonly used drug metformin. While, we are the first to show these effects of the aglycone naringenin in vitro, future studies to understand the mechanisms, safety and dose using primary cells lines and animal models are warranted before translating to dietary recommendations. In conclusion, we consider these pre-clinical studies to be important for improving our understanding of mechanisms of anti-diabetic effects of these bioactive compounds.
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APPENDIX A

<table>
<thead>
<tr>
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<th>CON</th>
<th>CLA</th>
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<tr>
<td>Muscle TG (mg TG/mg protein)</td>
<td>0.25 ± 0.04</td>
<td>0.20 ± 0.02</td>
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<td>Serum TG (mg/dl)</td>
<td>112.74 ± 11.93</td>
<td>125.7 ± 22.25</td>
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<tr>
<td>NEFA (mEq/L)</td>
<td>0.72 ± 0.10</td>
<td>0.70 ± 0.10</td>
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<tr>
<td>*CPT-I mRNA</td>
<td>1.0 ± 0.28</td>
<td>1.11 ± 0.20</td>
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<tr>
<td>*CPT-II mRNA</td>
<td>1.0 ± 0.21</td>
<td>1.43 ± 0.19</td>
</tr>
<tr>
<td>*G-6-Pase mRNA</td>
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<td>1.1 ± 0.11</td>
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<tr>
<td>*PEPCK mRNA</td>
<td>1.0 ± 0.26</td>
<td>1.06 ± 0.15</td>
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Table A.1 Muscle, serum TG and serum NEFA
* Fold induction over control (target mRNA / 18s mRNA). Values represent mean ± SE.
APPENDIX B

Figure B.1 Muscle TG from study 2. Muscle triglycerides were measured from male ob/ob mice after two weeks on either control (CON) or CLA-supplemented (CLA) diets and injected with PBS or ROSI. Values represent mean ± SE.
Figure B.2 Effect of CLA on hepatic AMPK and ACC phosphorylation. Male C57BL/6 mice were fed a diet containing 1.5% CLA (+CLA, n=10) for four weeks followed by four weeks without CLA (-CLA, n=5). (A) p-AMPK (B) p-ACC measured using western blotting. Lanes 1 and 2, +CLA; lanes 3 and 4, -CLA. Vertical bars represent mean ± SD, ** P<0.05.
Table B.1 Body weights, organ weights and serum metabolites from female *ob/ob* mice (study 1) at baseline, after four weeks of feeding diet with CLA (+) and after an additional four weeks of feeding diet without CLA (-). Values represent mean ± SE. Superscripts represent significant differences between treatments, P<0.05.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>+CLA</th>
<th>-CLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>78.57 ± 1.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.72 ± 1.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.67 ± 3.81&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver Weight (% Bwt)</td>
<td>--</td>
<td>8.25 ± 1.5</td>
<td>5.49 ± 0.62</td>
</tr>
<tr>
<td>Liver TG (% Liver wt)</td>
<td>--</td>
<td>23.27 ± 3.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.01 ± 1.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AUC</td>
<td>--</td>
<td>11805.00 ± 2457.67</td>
<td>2837.50 ± 1221.7</td>
</tr>
</tbody>
</table>
Figure B.3 Effect of CLA on time course depletion/repletion of adiponectin from study 1. Female ob/ob mice were fed a diet containing 1.5% CLA (+CLA, n=6) for four weeks followed by four weeks without CLA (-CLA, n=3). Adiponectin concentrations were determined at indicated times. * P<0.05 vs. baseline, § P<0.05 vs. last time point on the diet containing CLA.
Table B.2 Body weight and tissue weights from male ob/ob mice fed CON or CLA diets for 4 weeks. Values represent mean ± SE. Superscripts represent significant differences between groups, p<0.05.

<table>
<thead>
<tr>
<th>Weights (g)</th>
<th>CON</th>
<th>CLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight</td>
<td>42.20 ± 0.97(^a)</td>
<td>28.98 ± 0.97(^b)</td>
</tr>
<tr>
<td>Change in body weight</td>
<td>9.74 ± 0.56(^a)</td>
<td>-3.54 ± 0.56(^b)</td>
</tr>
<tr>
<td>Epididymal adipose</td>
<td>2.96 ± 0.14(^a)</td>
<td>1.71 ± 0.14(^b)</td>
</tr>
<tr>
<td>Liver</td>
<td>2.76 ± 0.17(^a)</td>
<td>3.18 ± 0.17(^b)</td>
</tr>
</tbody>
</table>
Figure B.4 Body weight over time in male ob/ob mice (A) Body weight, (B) Weight Gain. Male ob/ob mice were fed CON (6.5% soybean oil) or CLA (1.5% CLA plus 5% soybean oil) diet for 4 weeks. Values represent mean ± SE, * P<0.05.
Table B.3: Serum metabolites in male ob/ob mice fed CON or CLA diets for 4 weeks.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CON</th>
<th>CLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (pmol/L)</td>
<td>441.24 ± 49.45	extsuperscript{a}</td>
<td>771.05 ± 58.51	extsuperscript{b}</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>9.37 ± 0.57	extsuperscript{a}</td>
<td>3.91 ± 0.57	extsuperscript{b}</td>
</tr>
<tr>
<td>Resistin (ng/ml)</td>
<td>22.13 ± 2.20	extsuperscript{a}</td>
<td>14.67 ± 2.20	extsuperscript{b}</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>88.13 ± 14.42	extsuperscript{a}</td>
<td>131.45 ± 14.42	extsuperscript{b}</td>
</tr>
<tr>
<td>IL-6 mRNA (fold induction)*</td>
<td>1.15 ± 0.22</td>
<td>1.24 ± 0.30</td>
</tr>
<tr>
<td>TNF-α mRNA (fold induction)*</td>
<td>1.0 ± 0.09</td>
<td>1.5 ± 0.09</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline</td>
<td>115.12 ± 10.38</td>
<td>101.25 ± 10.38</td>
</tr>
<tr>
<td>2 weeks</td>
<td>130.25 ± 10.38	extsuperscript{a}</td>
<td>161.59 ± 12.10	extsuperscript{b}</td>
</tr>
<tr>
<td>4 weeks</td>
<td>122.87 ± 10.38	extsuperscript{a}</td>
<td>153.63 ± 10.38	extsuperscript{b}</td>
</tr>
</tbody>
</table>

	extsuperscript{a,b} Values represent mean ± SE with significant differences (P<0.05). 
	extsuperscript{#} Values represent mean ± SE with significant differences (P<0.05) from baseline.

* Adipose mRNA normalized to 18s.
Figure C.1 Combination of insulin and naringenin do not have additive effects on suppression of HGP from Fao cells. Cells were incubated in GNG medium for 3hr in the presence of Dmso (Veh), insulin (IN, 25nM), naringenin (N, 100µM) and insulin plus naringenin (IN 25nM + N 100µM). Glucose concentration in the medium was measured using Amplex red glucose oxidase kit (Molecular probes). Values represent mean ± SE, superscripts represent significant differences between treatments, p< 0.05.
Figure C.2 Insulin but not naringenin or metformin activate Akt in Fao cells. (A) 3hr; (B) 9hr. Insulin (IN, 100nM) increased phosphorylation of the Ser 473 residue (hashed bars) compared to Veh treated cells. Phosphorylation of the Thr 308 (solid bars) residue was unaffected by any treatment. Treatments were in GNG medium in the presence of cAMP + dex for 6hr. Lanes: 1,2 Veh; 3,4 naringenin; 5,6 insulin; 7,8 metformin. Values represent mean ± SEM. Superscripts represent significant differences between treatments for the Ser 437 residue, P<0.05.
Figure C.3 Induction of G-6Pase mRNA in Fao cells. Treatment cAMP + dex does not induce mRNA levels of G-6Pase in Fao cells treated for 4hr in GNG medium.
Figure C.4 Effect of naringenin on cell viability and protein concentration. Fao cells were incubated in GNG medium with treatments. (A) Cell viability was determined using trypan blue exclusion method after 6hr; (B) Protein concentration at indicated time points determined using BCA protein assay. C, control; Veh, DMSO; M, 1mM metformin.