A Dissertation

entitled

Hepatic CEACAM1 Protects Against Metabolic Abnormalities Associated with Metabolic Syndrome

by

Thomas A. Bowman

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________________________________________
Dr. Sonia M. Najjar, Major Advisor

________________________________________
Dr. Maurice Manning, Committee Member

________________________________________
Dr. Raymond E. Bourey, Committee Member

________________________________________
Dr. Beata Lecka-Czernik, Committee Member

________________________________________
Dr. Sandrine V. Pierre, Committee Member

________________________________________
Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

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Impaired hepatic insulin clearance causes hyperinsulinemia and secondary insulin resistance, which may progress to involve various components of metabolic syndrome. The carcinoembryonic-related cell adhesion molecule 1 (CEACAM1) has been shown to promote insulin clearance, downregulate the mitogenic action of insulin, and limit lipogenesis in the early hours of refeeding. Mice with liver-specific Ceacam1 inactivation (L-SACC1) or with global null mutation (Cc1−/−), exhibit impairment of insulin clearance and hyperinsulinemia, which causes insulin resistance. Since the lack of CEACAM1 correlates with insulin resistance, and regulates insulin action in liver, an important diet-responsive organ, we proposed that reduction of CEACAM1 is implicated in the pathogenesis of diet-induced insulin resistance; and that increasing hepatic levels of CEACAM1 would be protective against metabolic abnormalities associated with metabolic syndrome. Therefore we examined CEACAM1 levels in an animal model of metabolic syndrome and non-alcoholic steatohepatitis (NASH), the low aerobic capacity runner (LCR) rats, in comparison to high aerobic capacity runner (HCR) rats. We found
that in response to caloric restriction by 30% over a period of 2-3 months profound improvements in insulin sensitivity and reversal of hepatic inflammation, oxidative stress and fibrosis. Caloric restriction exerts these effects along with increases in fasting levels of CEACAM1 in liver. Additionally we examined the effect of high-fat diet on wild-type mice and on a transgenic mouse with liver-specific overexpression of rat CEACAM1 (L-CC1). We found that an early event associated with high-fat feeding is repression of CEACAM1 by a PPARα-mediated mechanism, and that this leads to impaired insulin clearance prior to the development of a pro-inflammatory state. Transgenic overexpression of CEACAM1 in liver prevents hyperinsulinemia and insulin resistance, and limits visceral obesity and the metabolic response to high-fat intake.
Dedication

To my wife, Julie, I dedicate this work, and the rest of my life. May we enjoy together what God and we have accomplished in this educational endeavor. I wish to also dedicate this work to my major advisor, Sonia Najjar, for teaching me how to be a scientist; and to my parents, siblings (Rob, John, Anne, Peggy, Joe, and Tim) and daughters (Katie and Leanna).
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# Table of Contents

Abstract iii  
Dedication v  
Acknowledgements vi  
Table of Contents vii  
List of Abbreviations ix  
Chapter 1: Introduction 1  
1.1: Metabolic Syndrome 1  
1.2: Insulin Action 2  
1.3: Insulin Resistance 3  
1.4: Dyslipidemia 6  
1.5: Molecular mechanisms of Dyslipidemia 7  
1.6: High Fat Diet 11  
1.7: Fatty Liver 14  
1.8: Caloric Restriction 15  
1.9: Exercise 16  
1.10: The Mechanism of Action of CEACAM1 17  
1.11: Animal Models of Altered CEACAM1 17  
1.11.1: LSACC1 17  
1.11.2: C1−/− 18
Chapter 2: Caloric restriction reverses hepatic insulin resistance and steatosis in rats with low aerobic capacity

2.1: Abstract
2.2: Introduction
2.3: Materials and Methods
2.4: Results
2.5: Discussion
2.6: Acknowledgements
2.7: References

Chapter 3: CEACAM1 Repression by PPARalpha as an Obligate Step in Dietary Insulin Resistance

3.1: Abstract
3.2: Introduction
3.3: Research Design and Methods
3.4: Results
3.5: Discussion
3.6: Acknowledgements
3.7: References

Chapter 4: Discussion

Chapter 5: Summary

References
List of Abbreviations

CEACAM1  CarcinoEmbryonic Antigen-related Cell Adhesion Molecule 1
Ceacam1  gene encoding CEACAM1 protein
Cc1^{−/−}  global Ceacam1 null mouse
L-SACC1  liver-specific S503A CEACAM1 mutant mouse
L-CC1  liver-specific wild-type CEACAM1 overexpressing mouse
BL6  C57BL/6 genetic background
PPARα  peroxisome proliferator-activated receptor α
PPARα^{−/−}  null mutant PPARα mouse
Wy  Wy14,643 PPARα agonist
RD  regular diet
HF  high fat diet
FFA  free fatty acids
FATP-1  fatty acid transport protein-1
FAS  fatty acid synthase
PI3K  phosphoinositol-3-kinase
MAPK  mitogen activated protein kinase
LCR  low capacity runner
HCR  high capacity runner
AL  ad libitum
CR  caloric restriction
S  sedentary
T  trained
TBARS  Thiobarbituric acid reactive substances
IPGTT  intraperitoneal glucose tolerance test
ITT  insulin tolerance test
Ib  immunoblot
Relb  reimmunoblot
WAT  white adipose tissue
H&E  hematoxylin and eosin
ECL  enhanced chemi-luminescence
IR  insulin receptor
IRS  insulin receptor substrate
GLUT4  glucose transporter-4
tyrosine^{1316}  tyrosine at amino acid position 1316
tyrosine^{488}  tyrosine at amino acid position 488
NAFLD  non-alcoholic fatty liver disease
NASH  non-alcoholic steatohepatitis
Chapter 1

Introduction

1.1 Metabolic Syndrome

The "metabolic syndrome" is a cluster of metabolic diseases that occur concurrently, that include at least 3 of 5 risk factors for the development of cardiovascular disease and type 2 diabetes mellitus from the National Cholesterol Education Program Adult Treatment Panel III definition (Grundy et al., 2005; Lloyd-Jones et al.). These risk factors include fasting plasma hyperglycemia, low levels of plasma high density lipoproteins, high levels of plasma triglycerides, high waist circumference, and high blood pressure (Grundy et al., 2005; Lloyd-Jones et al.). Some defined the metabolic syndrome as "insulin resistance syndrome" since insulin resistance is an essential component of the originally defined syndrome (Reaven, 1988). The prevalence of the metabolic syndrome in the United States was approximately one-third of all adults (Lloyd-Jones et al.). Therefore, the metabolic syndrome contributes significantly to the incidence of morbidity of both cardiovascular disease and diabetes mellitus. Sequelae and a significant correlation with the metabolic syndrome include non-alcoholic fatty liver disease (NAFLD) and its advanced form of nonalcoholic steatohepatitis (NASH) (Abdeen et al., 2006; Marchesini et al., 2001).
1.2 Insulin Action

Insulin is a peptide hormone produced in the β-cells of the pancreas. Insulin is an anabolic hormone that is produced in response to feeding. Nutrients such as glucose and amino acids cause pancreatic insulin secretion. Insulin is secreted in a pulsatile manner into the portal vein and the liver by the "first-pass" effect clears approximately half of the insulin (Duckworth et al., 1998). Its anabolic action promote storage of nutrients, including glucose uptake and inhibition of lipolysis of adipose tissues. Insulin action depends on its binding to the insulin receptor at a ratio of one or two insulin molecules for each insulin receptor. Insulin binds to one of the two α-chains on the extracellular portion of the receptor on the plasma membrane of insulin target tissues, including muscle, liver and adipose. After binding of insulin to the insulin receptor, the two intracellular β-chains of insulin receptor autophosphorylate and phosphorylate and bind to intracellular substrate molecules through several tyrosine kinase residues. It is through these insulin receptor substrates that insulin produces its various effects through two canonical pathways (see Figure 1.1): phosphoinositol-3-kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway is important for cell division. The PI3K pathway is an important pathway in promoting storage of nutrients, including insulin-stimulated glucose uptake. The PI3K pathway is also important in regulating the fasting to feeding transition, whereby insulin acutely inhibits gluconeogenesis and fatty acid synthesis (Najjar et al., 2005a; Saltiel, 2005) in the liver and induces translocation of GLUT4 for glucose uptake and utilization in muscle. Insulin also inhibits fatty acid lipolysis. These changes occur so that glucose is utilized by insulin
responsive tissues during feeding hours, whereas when insulin is low, during fasting, fatty acids are utilized.

Figure 1.1: Insulin signaling pathways and functions. Shown in this figure are the primary two pathways downstream of insulin binding the insulin receptor. The pathway on the right is the MAPK pathway, important for cell division. The PI3K pathway on the left produces most of insulin's metabolic affects.

1.3 Insulin Resistance

Insulin resistance is defined as an inability of insulin to bind to and activate its receptor. Insulin resistance primarily effects its roles through the PI3K pathway. When insulin resistance occurs, gluconeogenesis during feeding is not suppressed and glucose uptake in muscle is ineffective. Insulin resistance is associated with increased fasting plasma levels of insulin (hyperinsulinemia). The cause-effect relationship of insulin resistance and hyperinsulinemia is complex: resistance to insulin binding its receptor may trigger compensatory insulin secretion producing hyperinsulinemia (Polonsky et al., 1988; Valera Mora et al., 2003). Alternatively, hyperinsulinemia may precede and initiate
the insulin resistance by ligand-induced down-regulation of receptors. One mechanism of hyperinsulinemia preceding insulin resistance is by primary impairment of insulin clearance (Escobar et al., 1999; Polonsky et al., 1988) (see Figure 1.2). When there is a primary impairment of insulin clearance this leads to hyperinsulinemia, which causes increased production of lipogenic enzymes, including acetyl-coenzyme A carboxylase and fatty acid synthase (Assimacopoulos-Jeannet et al., 1995; Ide et al., 2004; Kersten, 2001; McCormick et al., 1979). Hyperinsulinemia stimulated lipogenesis of liver may produce fatty liver and/or increased plasma triglycerides. The compensation for increased plasma triglycerides is increased insulin secretion to promote storage of triglyceride in white adipose tissue. White adipose tissue remains insulin sensitive with even high levels of insulin. However, with expansion and saturation of white adipose tissue (WAT) (Freedland, 2004), insulin repression of lipolysis is inadequate to prevent an increase in release of free fatty acids (FFA) (Collin et al., 2006). The increase in FFA release from WAT in obese states further reduces insulin clearance by activation of novel PKC isoforms (Chen et al., 2006; Lam et al., 2002; Samuel et al., 2004) and by downregulation of insulin receptors in liver (Svedberg et al., 1990; Svedberg et al., 1991). This FFA induced impairment of insulin clearance exacerbates insulin resistance in liver requiring more compensatory insulin secretion.
Figure 1.2: Schematic of physiology of insulin resistance caused by primary impairment of insulin clearance.

FFA induced persistent insulin resistance may lead to β-cell exhaustion (Lewis et al., 2002) and insufficiency of insulin secretion to overcome resistance (Leung et al., 2004). The flux of triglyceride to muscle produces increases in intramyocellular lipids and their metabolites, such as fatty acyl CoAs and diacylglycerol. These metabolites activate serine/threonine kinases in muscle, which impair insulin signaling through serine/threonine phosphorylation of insulin receptor substrate (IRS)-1 (Morino et al., 2006). Plasma FFA released from WAT also contribute to lipid accumulation in hepatocytes. Additionally, liver insulin resistance produces an overproduction of triglycerides resulting in fatty liver and if triglyceride export is intact, hypertriglyceridemia. Insulin resistance in adipose tissue, produces reduced inhibition of lipolysis, resulting in lipid accumulation in non-adipose tissues. Type 2 diabetes mellitus involves the progression of insulin resistance to produce both hyperglycemia and hyperinsulinemia.
1.4 Dyslipidemia

Dyslipidemia is the pathological accumulation of lipids in the blood stream. The important lipids in the pathogenesis of disease states include triglycerides, cholesterol and lipoproteins of different densities. High levels of cholesterol and particularly of low-density lipoproteins increase the risk of morbidity and mortality. Conversely, high-density lipoproteins inversely correlate with an increased risk of morbidity and mortality.

The prevalence of dyslipidemia is only recently decreasing in the United States due to increasing use of lipid lowering medications (Gregg et al., 2005). However, quite the opposite is the case with the rates of two disorders consequent to dyslipidemia, obesity and type 2 diabetes mellitus, which continue to rise. The most immediately life threatening consequence of dyslipidemia is cardiovascular disease. Cardiovascular disease is the primary worldwide cause of death. In the eastern world, dyslipidemia is on the rise due to the export of the western diet, and industrialization causing a decrease in exercise. Asians also have a higher risk of comorbidities from dyslipidemia and being overweight. This may be due to higher percentage body fat and a high incidence of cigarette smoking among Asians. Consequently, the World Health Organization (WHO) recommended in 2003 lower body mass index and lipid levels for Asians. The WHO estimated the number of cigarette smokers in 2003 to be 1.3 billion, which has contributed to cardiovascular disease at least in part due to lipid toxicities from tobacco use (2003).

It takes only a mild elevation of triglycerides to increase morbidity from cardiovascular disease (Fung and Frohlich, 2002). The National Cholesterol Education Program-Adult Treatment Panel-III made the following recommendations (Grundy et al.,
2005; Lloyd-Jones et al.): (1) LDL cholesterol optimal level is less than 100 mg/dL; (2) total cholesterol desirable level is less than 200 mg/dL; (3) HDL cholesterol should be 40 mg/dL or greater, and less than 60 mg/dL; (4) triglyceride should be less than 150 mg/dL. Therapeutic options recommended to control dyslipidemia include primarily weight loss, dietary restrictions, exercise, cessation of smoking, and pharmaceuticals including aspirin, cholesterol lowering drugs and statins. Secondary disease states will also need observation and control including cardiovascular disease, diabetes, hypertension and peripheral arterial disease (Fung and Frohlich, 2002).

1.5 Molecular mechanisms of Dyslipidemia

The primary function of the liver is to either filter or buffer the body from the toxic effects of a multitude of endogenous and exogenous substances. Furthermore, the liver converts many active and harmful substances to inactive and harmless substances through catabolism. It is noteworthy that some catabolic products are actually more deleterious than their substrates. There are four nutrient related catabolic pathways in the liver: glycogenolysis, lipolysis, glycolysis, and cholesterol metabolism. Conversely, there are four reciprocal pathways for anabolism: glycogenesis, gluconeogenesis, lipogenesis, and cholesterol synthesis.

Glucose plays a central role in all the metabolic pathways of hepatocytes because it is a basic substrate in the body and the primary energy-producing nutrient in the human diet. The human gut microbiota metabolizes most dietary carbohydrates into this basic energy source and further directs the liver to increase lipogenesis (Backhed et al., 2004). Dietary glucose effects a multitude of hormone levels, including directly on insulin secretion from β-cells of the pancreas (Dai et al., 2004); and also indirectly on
hypothalamic anorexigenic neuropeptides through ghrelin secretion from the stomach (Hu et al., 2005).

Chronic hyperinsulinemia is a lipogenic stimulus, which increases production of enzymes acetyl-coenzyme A carboxylase and fatty acid synthase (Assimacopoulos-Jeannet et al., 1995; Ide et al., 2004; Kersten, 2001; McCormick et al., 1979). This hyperinsulinemia stimulated lipogenesis of liver may produce fatty liver and/or increased plasma triglycerides. However, the compensation for increased plasma triglycerides is increased insulin secretion to promote storage of triglyceride in white adipose tissue. White adipose tissue remains insulin sensitive with even high levels of insulin. Yet with expansion and saturation of white adipose tissue (WAT) (Freedland, 2004), insulin repression of lipolysis is inadequate to prevent an increase in release of free fatty acids (FFA) (Collin et al., 2006). In this manner, chronic hyperinsulinemia contributes to dyslipidemia. Conversely, acute pulses of insulin actually repress hepatic lipogenesis (Najjar et al., 2005a).

Hormones other than insulin also regulate lipids. For example, growth hormone opposes dyslipidemia by decreasing insulin sensitivity in adipose tissue and by inducing growth and development of muscle which is able to burn fat (Kersten, 2001). However, growth hormone produces increased intramuscular fatty acid oxidation at the expense of glucose metabolism (Bramnert et al., 2003). Additionally, leptin produced by adipocytes opposes lipogenesis and adipogenesis, and favors lypolyis. Leptin is produced proportionally to the growth of adipose tissue mass and is a primary means of homeostatic control of adiposity (Kersten, 2001; Mora and Pessin, 2002).
In the fasting state lipogenesis decreases due to lower insulin levels. However, with prolonged fasting, fatty acids in large amounts escape from adipose tissues due to low insulin control of hormone sensitive lipase. As a result liver triglycerides will actually increase due to utilization of excessive free fatty acids from the plasma (Kersten, 2001).

Glucose activates lipogenesis. A recent study demonstrated activation of the carbohydrate-responsive transcription factor of lipogenesis, carbohydrate response element binding protein (ChREBP), through phosphatase (PP2A) activation through the glycolysis shunt product xylulose 5-phosphate (Kabashima et al., 2003). Additionally, glucose action through ChREBP is opposed by cyclic AMP repression of lipogenesis (He et al., 2004; Kawaguchi et al., 2001).

Lipid metabolism in the liver is predominantly through the nuclear hormone related family of receptors. These bind a ligand and form a heterodimer, usually with RXR, translocate to the nucleus and bind to DNA nuclear response elements through their conserved DNA binding domains containing zinc finger motifs. There is significant cross-talk between the receptor pairs due to competition for RXR dimerization. This cross-talk produces antagonism between peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) (Yoshikawa et al., 2003).

Transcription factors from the basic helix-loop-helix zipper family promote lipogenesis and cholesterol synthesis. Three members of this family are in the liver: (1) The steroid regulatory element binding protein-1c (SREBP-1c) codes for triglyceride synthesis through fatty acid synthase and is inhibited primarily by polyunsaturated fatty acids. (2) The carbohydrate response element binding protein (ChREBP) targets
lipogenesis through acetyl CoA carboxylase and is inhibited primarily by Glucagon (via repression cyclic AMP).  (3) The SREBP-2 targets cholesterol synthesis through primarily HMG-CoA Reductase and HMG-CoA Synthase, squalene synthase, and low-density lipoprotein receptor (LDLR).  SREBP-2 functions as a nuclear transcription factor by cleavage and subsequent nuclear translocation of its N-terminal by SREBP Cleavage-Activating Protein (SCAP) and is inhibited by binding of Activating Transcription Factor 6 (ATF6). (Fatehi-Hassanabad and Chan, 2005; Lu et al., 2001; Towle, 2001; Zeng et al., 2004)

A primary inhibitor of dyslipidemia is the peroxisome proliferator activated receptor (PPAR). PPARs were named due to their activation by peroxisomal proliferators, such as fibrates and Wy-14,643 (Green, 1995; Mukherjee et al., 1994; Reddy, 2004). PPARs also are activated by fatty acids (Fatehi-Hassanabad and Chan, 2005; Gottlicher et al., 1992), particularly by newly synthesized and dietary fatty acids (Chakravarthy et al., 2005b; Hsu and Huang, 2007). There are two predominant PPARs that regulate fatty acids: PPARα and PPARγ. The liver contains predominantly PPARα, which acts as transcription factor on PPAR response elements (PPRE) to upregulate genes important for fatty acid and cholesterol oxidation, such as carnitine palmitoyltransferase-I (CPT-1), apolipoprotein-A1 (ApoA1), and CD36, a fatty acid transporter (Fatehi-Hassanabad and Chan, 2005). PPARα also downregulates genes important for lipogenesis, such as malic enzyme (Fatehi-Hassanabad and Chan, 2005). The adipose contains predominantly PPARγ, which acts as a primary transcription factor for differentiation of adipocytes (Rosen et al., 1999) and for increased fatty acid storage as triglyceride, increased insulin sensitivity, and decreased inflammation (Fatehi-
Hassanabad and Chan, 2005). The combined effect of PPARs is therefore to decrease blood content of fatty acids to decrease lipotoxicity.

1.6 High Fat Diet

Diets high in fat content are among the most studied inducers of obesity and insulin resistance. The high fat diet contributes to increased rates of obesity due in part to having a dense energy content (Hensrud, 2004; Lichtenstein et al., 1998). Dietary fatty acids are able to incorporate into tissue membranes including adipose tissue (van Staveren et al., 1986) and erythrocyte membranes (Katan et al., 1991) in direct proportion to the content in the diet. Chronic intake of high levels of fatty acids produces insulin resistance (Hunnicutt et al., 1994) and increased adiposity (Doucet et al., 1998). Dietary fatty acids are transported from the small intestine in the circulation by chylomicrons. Whether transported as free fatty acids or esterified into triglyceride, these fatty acids leave chylomicrons equally to enter the tissues of the body (Hultin et al., 1996).

Chronic intake of unsaturated fatty acids impair the ability of pancreatic beta cells to secrete insulin in response to glucose, whereas saturated fatty acids may enhance insulin secretion, although not enough to overcome peripheral insulin resistance (Dobbins et al., 2002). The impairment of glucose stimulated insulin secretion from high fat diet occurs in parallel to the increased triglyceride content of pancreatic islets (Eto et al., 2002). The resultant effect of fatty acids and triglycerides on beta cells is chronic fasting hyperinsulinemia and reduced insulin secretion in response to glucose.

Additionally, fatty acids cause hepatic impairment of insulin clearance (Bergman, 2000; Svedberg et al., 1991; Yoshii et al., 2006) that increases hyperinsulinemia. This impairment of insulin clearance is a partial compensatory response in order to improve
insulin action in peripheral insulin resistant tissues (Mittelman et al., 2000). Since insulin is secreted into the portal vein, the liver is ideally located to regulate insulin clearance in response to these dietary stimuli. Interestingly, PPARα null mice remain insulin sensitive and are protected from hyperinsulinemia after even a prolonged high-fat diet (Guerre-Millo et al., 2001). Since PPARα is primarily found in liver, and since the liver is a primary site of insulin clearance, this protection from hyperinsulinemia implies that PPARα regulates insulin clearance in response to high fat feeding.

This high fat diet-induced chronic hyperinsulinemia increases lipid uptake into muscle, which reduces glucose uptake (Agus et al., 2000) and produces insulin resistance except in skeletal muscle of trained endurance athletes, which has an increased capacity to oxidize lipids (Goodpaster et al., 2001; Thamer et al., 2003). However, muscle lipid accumulation does not directly reduce glucose uptake (Krebs and Roden, 2005). A long held view was described by Randle and colleagues that increased fatty acids produced greater fatty acid oxidation in muscle, resulting in citrate inhibition of glycolysis, which prevented glucose uptake by competition (Garland et al., 1963; Randle et al., 1963). Recent experiments challenged these views, proposing that accumulation of lipid metabolites, fatty acyl CoAs, ceramides and diacylglycerol, activate novel PKC isoforms that act in muscle as serine/threonine kinases that impair insulin signaling through inactivation of insulin receptor substrate (IRS)-1 (Corcoran et al., 2007; Morino et al., 2006); this blunting of PI3K/Akt signaling would decrease glucose transporter (GLUT)-4 translocation to the sarcolemma. Fatty acids also appear to produce a similar mechanism of insulin resistance in liver, involving novel PKC isoforms (Chen et al., 2006; Lam et
al., 2002; Samuel et al., 2004) inactivating IRS, resulting in hyperinsulinemia and
elevation of hepatic glucose production.

High fat diet-induced obesity with insulin resistance is associated with the
accumulation of inflammatory cells, including macrophages in white adipose tissue
(WAT) (Strissel et al., 2007; Xu et al., 2003), and with inflammation systemically in
many other tissues, including liver (Boden, 2006; Cai et al., 2005), vasculature (Kim et
al., 2008a; Li et al., 2007; Sjoholm and Nystrom, 2005), and muscle (Boden, 2006;
Hotamisligil et al., 1996; Oakes et al., 1997). Additionally these studies link
improvement in insulin sensitivity by blocking this inflammation. Nevertheless, it is
unclear the cause/effect relationship of inflammation and insulin resistance. Certainly, the
evidence shows that inflammation can cause insulin resistance. However, diet-induced
insulin resistance occurs in the early stages of weight gain before the development of
significant obesity or inflammation, occurring primarily due to hyperinsulinemia from
decreased insulin clearance (Erdmann et al., 2008). Furthermore, macrophage
accumulation with the appearance of crown-like structures in histological sections in
WAT and of a significant increase in inflammatory markers (tumor necrosis factor
(TNF)-alpha, monocyte chemotactic protein (MCP)-1, and interleukin (IL)-10 and IL-6
proteins) appear starting at approximately 12 to 16 weeks of high-fat feeding (Kim et al.,
2008a; Strissel et al., 2007), much later than that of high-fat diet induced glucose
intolerance in rats, which occurred at 14 days of feeding (Chanseaume et al., 2007), and
later than that of high-fat diet induced insulin resistance in mice, which occurs
approximately 3 to 8 weeks of feeding (Kim et al., 2008a; Park et al., 2005a).

Furthermore, although TNFα is a critical mediator of inflammation, activation of its
receptor is not necessary for high-fat diet-induced obesity and insulin resistance to develop (Pamir et al., 2009). Additionally, mice lacking T lymphocytes (Rag1−/−) become insulin resistant and obese when fed a high fat diet (Winer et al., 2009). In summary, the evidence points to inflammation as a secondary contributor to insulin resistance that occurs subsequent to a primary impairment of insulin clearance, hyperinsulinemia, and dyslipidemia.

1.7 Fatty Liver

While visceral obesity is a marker of the metabolic syndrome, fatty liver is more closely linked to insulin resistance (Fabbrini et al., 2009), since it is more directly involved in dyslipidemia. Visceral adipose tissue is capable of accumulating substantial triglyceride without any systemic pathology, since visceral adipose tissue remains insulin sensitive, resistant to increased lipolysis for the first few weeks of high-fat feeding, whereas intrahepatic fat accumulates within the first week of feeding (Collin et al., 2006; Gauthier et al., 2006). Conversely, significant pathology arises when triglyceride is stored ectopically, such as in liver. Hepatic triglyceride accumulation progresses to steatohepatitis and diabetes and is significantly correlated to mortality due to cardiovascular disease, and the incidence of obesity and end-stage liver disease, including hepatocellular carcinoma (Ekstedt et al., 2006; Soderberg et al., 2010).

The accumulation of triglyceride in liver that is not due to alcohol ingestion is termed "non-alcoholic fatty liver disease (NAFLD)." This disease state is a reflection of 1) an increased dietary delivery of fatty acids to the liver (Chakravarthy et al., 2005b; Hensrud, 2004; Kim et al., 2007; Lichtenstein et al., 1998), 2) a decreased fatty acid oxidative capacity (Gerhart-Hines et al., 2007; Leone et al., 1999; Matsuzawa-Nagata et
al., 2008), 3) elevated de novo synthesis of fatty acids (Anderson and Borlak, 2008; Schutz, 2004; Schwarz et al., 2003), 4) impaired lipoprotein secretion/export (Anderson and Borlak, 2008; Schutz, 2004; Schwarz et al., 2003), and 5) peripheral insulin resistance (Lewis et al., 2002). Progression of NAFLD to non-alcoholic steatohepatitis (NASH) has been described as a "two-hit" mechanism (Day and James, 1998) in which the first "hit," triglyceride accumulation, does not progress to NASH without a second "hit," such as oxidative stress or inflammation. As described above (Section 1.6), inflammation appears to follow hepatic triglyceride accumulation (Cai et al., 2005) and insulin resistance (Erdmann et al., 2008). So this "two-hit" model of NASH fits well with high-fat feeding models of inflammation as a secondary contributor to insulin resistance that occurs subsequent to a primary impairment of insulin clearance, hyperinsulinemia, and dyslipidemia as discussed in the previous section.

1.8 Caloric Restriction

Caloric restriction alone or in combination with exercise is effective for treatment of obesity and obesity related diseases and for extending lifespan (Guarente, 2008; Larson-Meyer et al., 2008; Redman and Ravussin, 2009; Ross et al., 2000). Caloric restriction produces salutary effects in large part by improving insulin sensitivity and decreasing fasting plasma insulin levels (Park et al., 2005c; Zhu et al., 2005). For example, one study showed that exogenous insulin reverses reductions from caloric restriction of reactive oxygen and lipid peroxidation (Lambert et al., 2004). Accordingly, caloric restriction reduces hepatic injury and lipid peroxidation despite increasing PPARα dependent mobilization and oxidation of fatty acids (Corton et al., 2004; Lopez-Lluch et al., 2006). Consequently, caloric restriction increases mitochondrial efficiency and biogenesis.
(Lopez-Lluch et al., 2006; Rodgers et al., 2005) while reducing hepatic lipid content (Larson-Meyer et al., 2008) and hepatic lipogenesis (Zhu et al., 2004). Also, in agreement with findings of improved energetic efficiency and insulin sensitivity, caloric restriction improves muscle (Arias and Cartee, 2007) and adipose tissue (Park et al., 2005c) glucose transport. Caloric restriction also inhibits induction of plasma inflammatory cytokines in streptozotocin-induced diabetic rats (Ugochukwu and Figgers, 2006).

1.9 Exercise

Inactivity is a major contributor to metabolic and cardiovascular diseases (2003; Handschin and Spiegelman, 2008). Inactivity produces chronic diseases in part due to an increase in inflammation (Giannopoulou et al., 2005; Stewart et al., 2005). Genetic predisposition for low exercise aerobic capacity produces a phenotype equivalent to the metabolic syndrome (Bernal-Mizrachi and Semenkovich, 2006; Wisloff et al., 2005), but markers of the metabolic syndrome can be reversed by endurance training (Wisloff et al., 2005). Endurance training has many beneficial effects, particularly in improving cardiovascular health (Haram et al., 2009; Schjerve et al., 2008; Wisloff et al., 2005; Wisloff et al., 2007). A single bout of exercise can improve insulin sensitivity and glucose transport (Arias et al., 2007). Both forced and voluntary exercise produce inhibitory effects on food intake (Kawaguchi et al., 2005; Looy and Eikelboom, 1989; Oscai and Holloszy, 1969). However, it has been noted, that despite the obvious importance of exercise, recent studies have shown a lack of improvement by exercise in comparison to caloric restriction alone for reduction of abdominal fat in obese humans (Redman et al., 2007); plus endurance training of obese rats failed to reduce hyperinsulinemia (Christ et al., 2002; Fiebig et al., 2002), hepatic fatty acid synthase
mRNA levels (Fiebig et al., 2002) and did not improve muscle insulin receptor action despite improvement in glucose uptake (Christ et al., 2002).

1.10 The Mechanism of Action of Hepatic CEACAM1

The carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a substrate for phosphorylation by the insulin receptor tyrosine kinase (Najjar et al., 1995) in addition to having cell-adhesion, cancer, angiogenesis and inflammation modulating effects. It is ubiquitously expressed except for muscle and adipose tissue (Accili et al., 1986). The insulin receptor C-terminal tyrosine\textsuperscript{1316} is responsible for phosphorylation of CEACAM1 tyrosine\textsuperscript{488} (Soni et al., 2000). Subsequently, phosphorylated CEACAM1 targets the insulin receptor through the phosphorylated juxtamembrane tyrosine\textsuperscript{960} to clathrin coated vesicles for endocytosis and insulin degradation. Phosphorylated CEACAM1 also sequesters Shc to downregulate insulin stimulated cell growth, proliferation and survival (Poy et al., 2002a). Plus, phosphorylated CEACAM1 binds to fatty acid synthase to limit hepatic lipogenesis in response to acute pulses of insulin in the early hours of feeding. Binding fatty acid synthase reduces its enzymatic activity in a biphasic manner that mirrors insulin release and corresponding increases in phosphorylated CEACAM1 (Najjar et al., 2005a).

1.11 Animal Models of Altered CEACAM1

1.11.1 LSACC1

Liver-specific S503A-CEACAM1 transgenic mice (LSACC1) have hyperinsulinemia (Poy et al., 2002b). Due to mutation of serine\textsuperscript{503}, the insulin receptor cannot phosphorylate the tyrosine\textsuperscript{488}, producing a dominant negative CEACAM1 in liver. Consequently, LSACC1 mice have defective insulin clearance as measured by insulin
tolerance tests and by a metabolic clearance assay of plasma after injection of Iodine-125 labeled insulin (Poy et al., 2002b). Accordingly, hyperinsulinemia in LSACC1 mice causes whole body insulin resistance as measured by hyperinsulinemic-euglycemic clamp (Park et al., 2006), defective hepatic gluconeogenesis, glucose intolerance, visceral adiposity, and increased plasma free fatty acids and increased plasma and hepatic triglycerides (Poy et al., 2002b). This hepatic steatosis progresses to steatohepatitis by feeding LSACC1 mice a high-fat diet for 3 months (Lee et al., 2008).

1.11.2 CC1\(^{-/-}\)

Mice with homozygous CEACAM1 null mutation (CC1\(^{-/-}\)), like LSACC1, have impaired insulin clearance and hyperinsulinemia (DeAngelis et al., 2008a). Essentially, these mice duplicate the phenotype of LSACC1 mice. Hyperinsulinemic-euglycemic clamp effects on CC1\(^{-/-}\) mice revealed insulin resistance primarily in liver (DeAngelis et al., 2008a). Despite significant CEACAM1 expression in pancreatic β-cells, insulin secretion and insulin content of β-cells was found to be normal (DeAngelis et al., 2008a). Therefore, since CEACAM1 is expressed in many tissues, it is important to note that the effects observed in CC1\(^{-/-}\) on insulin action and metabolism are due to a lack of liver CEACAM1 and not extra-hepatic tissues.

1.11.3 LCR

Rather than rely only on monogenetic mutations in inbred mice in order to interpret the physiological role of CEACAM1 in humans, we also studied rats selectively bred for aerobic running capacity. These rats maintain genetic heterogeneity due to a rotational mating system that minimizes inbreeding. Since complex metabolic diseases depend by definition on multiple genes responding synergistically to environmental influences, this
animal model is better suited to replicate these conditions. Indeed, after several generations of selection, low capacity runners (LCR) and high capacity runners (HCR) differ in several markers of metabolic syndrome. Specifically, LCR in contrast to HCR have increased visceral adiposity, elevated plasma free fatty acids and triglycerides, hyperinsulinemia, insulin resistance, increased blood pressure (Wisloff et al., 2005), and a progressive form of hepatic steatosis, characterized by features of non-alcoholic steatohepatitis (NASH), including fibrosis and inflammation (Thyfault et al., 2009). Of particular interest to our research, LCR have markedly lower levels of CEACAM1 than HCR (Wisloff et al., 2005). In the following paper (Chapter 2), we have examined the beneficial effect of caloric restriction in the reversal of these metabolic abnormalities, with particular attention to the role of CEACAM1 in pathways that correlate to these beneficial effects.

1.11.4 LCC1

In the final paper (Chapter 3), we have introduced the liver-specific wild-type CEACAM1 transgenic mice (LCC1). In these mice overexpression of rat CEACAM1 in the liver of mice was accomplished similar to the design of the mutant transgenic LSACC1 by use of an ApoA1 promoter to drive liver-specific expression. Its expression levels were found to have 5 to 6 fold higher CEACAM1 in transgenic hemizygous progeny. The transgenic expression of wild-type CEACAM1 in liver protects these mice from high-fat diet induced metabolic abnormalities, which begin within 30 days of feeding. Furthermore, lifespan feeding of LCC1 mice for 31 weeks of high-fat diet revealed advanced long-term protection from impaired insulin clearance, hyperinsulinemia, impaired glucose uptake, glucose intolerance, visceral obesity,
hyperglycemia, and beta cell failure. These results validate the importance of hepatic CEACAM1 in metabolic pathways that control insulin target tissues and suggest that CEACAM1 would be a good therapeutic marker for control of metabolism in various disease states such as diabetes and metabolic syndrome.
Chapter 2

Caloric restriction reverses hepatic insulin resistance and steatosis in rats with low aerobic capacity

Thomas A. Bowman¹,², Meenakshi Kaw¹,²; Sang Jun Lee¹,², Payal R. Patel¹,², Raymond E. Bourey¹,³; Per Magnus Haram⁴,⁵, Lauren G. Koch⁶, Steven L. Britton⁶, Ulrik Wisløff⁵, Abraham D. Lee¹,⁷, and Sonia M. Najjar¹,²,†.

¹Center for Diabetes and Endocrine Research and Departments of ²Physiology and Pharmacology, ³Internal Medicine and ⁷Physical Therapy at the University of Toledo College of Medicine, Health Science Campus, Toledo, OH 43614; ⁴Department of Cardiothoracic and Vascular Surgery, University Hospital, North-Norway and Institute of Clinical Medicine, Faculty of Medicine, University of Tromsø; ⁵Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Trondheim, Norway; ⁶Department of Anesthesiology, University of Michigan, Ann Arbor, MI, 48109

†Address correspondence and reprint requests to:

Sonia M. Najjar, Ph.D.
College of Medicine
University of Toledo, Health Science Campus
3000 Arlington Avenue, Mail stop 1008
Toledo, Ohio, 43614
Tel: (419) 383-4183
FAX: (419) 383-2871
e-mail: sonia.najjar@utoledo.edu
Footnotes

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Short title: Caloric restriction in liver metabolism

Precis: This study provides new insights on the role of CEACAM1-dependent insulin clearance pathways in the reversal of altered lipid and insulin metabolism by caloric restriction.

Keywords: Caloric restriction, aerobic capacity, insulin clearance, CEACAM1, metabolic syndrome, NASH
2.1 Abstract

Rats selectively bred for low aerobic running capacity (LCR) exhibit the metabolic syndrome, including hyperinsulinemia, insulin resistance, visceral obesity and dyslipidemia. They also exhibit features of non-alcoholic steatohepatitis (NASH), including chicken-wire fibrosis, inflammation and oxidative stress. By comparison to rats with high running capacity (HCR), LCR exhibit a marked reduction in hepatic mRNA levels of the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a transmembrane glycoprotein, which upon its phosphorylation by the insulin receptor promotes receptor-mediated insulin uptake and degradation in liver. Consistently, hyperinsulinemia in LCR results from impairment of insulin clearance. Caloric restriction by 30% over a period of 2-3 months improves glucose and insulin tolerance in parallel to enhanced insulin clearance, and reduces visceral obesity together with serum and tissue (liver and muscle) triglyceride levels. It also reverses hepatic inflammation, oxidative stress and fibrosis. Caloric restriction appears to exert these effects, at least partly, by inducing protein content and insulin-stimulated phosphorylation of hepatic CEACAM1. The data lend further support to the significant role of caloric restriction in the reversal of metabolic derangements in liver.

2.2 Introduction

Low aerobic capacity constitutes a strong risk factor of mortality from metabolic diseases (Myers et al., 2002). We have shown that rats selectively bred for low aerobic running capacity via selection based on treadmill running distance to exhaustion (termed LCR) exhibit features of the metabolic syndrome, including visceral obesity, insulin resistance and high blood pressure, as compared to age-matched high capacity runners (HCR)
They also exhibit elevated hepatic and serum triglyceride levels with a progressive form of hepatic steatosis (Thyfault et al., 2009), characterized by features of non-alcoholic steatohepatitis (NASH), including chicken-wire fibrosis and inflammation (Abdeen et al., 2006; McClain et al., 2004).

Hyperinsulinemia in LCR is associated with impaired hepatic insulin clearance, which appears to correlate with reduced mRNA of the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (Wisloff et al., 2005). CEACAM1, a substrate of the insulin receptor kinase in liver, but not muscle or adipose tissue, promotes receptor-mediated insulin endocytosis and degradation, a process that underlies insulin clearance (Formisano et al., 1995). Consistent with the liver being the main site of endogenous insulin clearance, L-SACC1 mice with liver-specific inactivation of CEACAM1 develop hyperinsulinemia resulting from impaired insulin clearance (Poy et al., 2002b). This causes overall insulin resistance and altered glucose metabolism in liver, skeletal muscle and white adipose tissue (WAT), as demonstrated by hyperinsulinemic-euglycemic clamp analysis (Park et al., 2006b). L-SACC1 transgenics also develop hepatic steatosis, in agreement with the positive effect of insulin on lipogenesis in spite of insulin resistance (Brown and Goldstein, 2008; Haeusler and Accili, 2008). Increased hepatic triglyceride synthesis amplifies output as VLDL particles and redistribution to WAT to cause visceral obesity, and to skeletal muscle to alter lipid metabolism and glucose uptake (Park et al., 2006b). Moreover, elevation in hepatic steatosis activates resident macrophages, which together with infiltrated macrophages from WAT, triggers a pro-inflammatory state in liver and predisposes to the development of steatohepatitis in L-SACC1 mice (Lee et al., 2008). Mice with global Ceacam1 null mutation (Cct1+/–) also exhibit hyperinsulinemia,
whole body insulin resistance, liver steatosis and visceral obesity (DeAngelis et al., 2008a; Najjar et al., 2005a). Thus, the phenotype of L-SACC1 and Cc1−/− mice demonstrates that hyperinsulinemia causes insulin resistance and fat accretion in liver, followed by visceral obesity. The similarity in the phenotype of L-SACC1 and Cc1−/− mice emphasizes the role of hepatic insulin and fat metabolism in regulating the metabolic response in extra-hepatic tissues, and assigns a major role for CEACAM1 in this process.

Exercise and caloric restriction reduce co-morbidities of metabolic diseases. The recently completed 10-year follow-up of the diabetes prevention program achieved its most favorable results in the cohort of intensive lifestyle modification that maintained significant weight loss through a program of regular exercise and caloric restriction (2002; Goldberg et al., 2009). The mechanisms of the beneficial effect of regular exercise and caloric restriction are not well delineated, in part due to the limitation imparted by the paucity of human tissues for mechanistic analyses. To circumvent this problem, we subjected LCR, a replicate model of the human disease, to high-intensity interval endurance training for 8 weeks (Haram et al., 2009). This program effectively reduced cardiovascular risk, such as endothelial dysfunction and hypertension, fat mass and glucose intolerance (Haram et al., 2009). However, it did not reduce the hepatic protein content of fatty acid synthase (FAS), a key enzyme in de novo fatty acid synthesis (Haram et al., 2009). This suggests that endurance training does not suffice to reverse all metabolic abnormalities in LCR liver. Thus, we herein, examined whether caloric restriction plays a more beneficial role on hepatic lipid metabolism in LCR, and whether this implicates regulation of insulin clearance in liver.
2.3 Materials and Methods

Caloric restriction: Three month-old male LCR rats (n=20) from generation 20 were randomized to either caloric restriction (CR) or ad libitum (AL) feeding of regular rat chow (Lab Diet #5001) for up to 90 days (Nisoli et al., 2005). Initially, rats were placed on a 10% caloric restriction program for two weeks prior to a 30% caloric restriction program for the remainder of the study. Age-matched HCR were fed ad libitum throughout the studies.

Endurance training: Liver and serum samples were obtained from our previously used set of rats, which at 3 months of age, had been either left sedentary (S) or subjected to an aerobic interval endurance training for 8 weeks (T) (Haram et al., 2009). This high intensity interval training consisted of running for 1 hour/day, 5 days/week until VO$_{2\text{max}}$ reached stability. The animals were euthanized 48 hours post-exercise (Haram et al., 2009). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Glucose and insulin tolerance tests and blood glucose measurements: Glucose (IPGTT) and insulin (ITT) tolerance tests were measured after an overnight fast (1700h - 800h the next day for glucose tolerance, 1700h-1100h the next day for insulin tolerance). Rats were anesthetized, injected intraperitoneally with 0.50 U insulin/kg body weight (for ITT) or 2 g glucose/kg body weight (for IPGTT) and their whole venous blood from catheterized tail vein was drawn at 0-2h after injection to determine blood glucose levels. Automated “Accu-chek Aviva” test strips (Roche) were utilized for all glucose
measurements. Tests were repeated at 6 and 9 weeks of feeding in order to account for changes in body weight.

Metabolic analysis: Following an overnight fast, rats were anesthetized with sodium pentobarbital. Whole venous blood was drawn from the tail vein to measure serum insulin and C-peptide by radioimmunoassays (Linco Research), serum free fatty acids (FFA) by NEFA C kit (Wako) and serum triglycerides by Triglycerides reagent (Pointe). Liver tissue triglycerides were measured from livers harvested at end of treatments, homogenized in sucrose lysis buffer and separated by chloroform-methanol and aqueous sulfuric acid; the organic layer was dried, reconstituted by chloroform, dried and measured by Triglycerides reagent (Pointe), and normalized to protein content of lysates. Visceral adipose tissue was excised, weighed, and visceral adiposity expressed as percentage of total body weight.

Western blots: Livers were removed at sacrifice from sex-matched and age-matched rats after an overnight fast. We analyzed 20 µg of total lysates with 4-12 % SDS-PAGE prior to transferring proteins to nitrocellulose membranes. Proteins were immunoblotted (Ib) with polyclonal antibodies against CEACAM1 (α-CC1), FAS (α-FAS) (Najjar et al., 2005a) and CD36 (α-CD36, Santa Cruz), followed by re-immunoblotting (reIb) with monoclonal antibody against tubulin (Sigma) or with polyclonal antibody against α-actin (Santa Cruz) to normalize for total protein content. Following protein detection by ECL, protein bands were scanned and their density measured using Image J software, and calculated as percentage of the amount of proteins loaded.

Ex-vivo phosphorylation: Tissue lysates were treated with 100 nM insulin for 5 min prior to immunoprecipitation (Ip) with antibodies against phosphotyrosine (α-pY, Upstate
Biotechnology Inc), SDS-PAGE analysis and immunoblotting with polyclonal antibodies against phospho-CEACAM1 (α-pCC1, Bethel antibodies). The α-pCC1 affinity purified polyclonal antibody was raised in rabbit against a CEACAM1 phosphorylated peptide sequence flanking its unique Tyrosine 488 phosphorylation site: 482 KVDDVA (pY) TVLNF 493 (NCBI Accession AAK52601) (Najjar et al., 1995b).

Thiobarbituric acid reactive substances (TBARS) assay: Lipid peroxidation was measured as described (Lee et al., 2008) with modifications. Briefly, liver tissue was homogenized in 1.15% KCl before centrifugation at 10,000 rpm at 4 °C for 10 minutes. The supernatant (100 μl) was added to 8.1% SDS, 20% acetic acid, and 0.8% thiobarbituric acid, followed by heating at 95 °C for 60 minutes. n-butanol-pyridine (15:1) was added to the cooled mix prior to centrifugation at 4000xg for 10 minutes and the upper layer was removed to measure its absorbance at A532nm.

Histological analysis: Histological examination was established using hematoxylin-eosin (H&E) of formalin-fixed paraffin-embedded liver. For analysis of fibrosis, deparaffinized and rehydrated slides were also incubated in 0.1% solution of Sirius Red (Sigma, Direct Red 80) and mounted with resin, following manufacturer’s instructions.

Statistical analysis: Data were analyzed with SPSS software using one-way ANOVA with Bonferroni's Multiple Comparison Test, or Student’s t-test. P<0.05 were statistically significant. Bar and line graphs were produced by GraphPad Prism 4 software.

2.4 Results

Caloric restriction reduced body mass and visceral obesity in LCR: As previously reported (Wisloff et al., 2005), 6 month-old LCR males exhibited a 1.5-fold higher body
weight than their age-matched HCR counterparts (Table 2.1). This is partly attributed to elevated visceral obesity (by ~2-fold). Subjecting LCR to a 30% caloric restriction (CR) program reduced their body weight progressively to reach that of ad libitum-fed HCR (AL-HCR) after about 50-60 days (Figure 2.1 and Table 2.1, CR-LCR versus AL-HCR). Visceral obesity was also reduced by 50% by caloric restriction to become comparable to that in AL-HCR (Table 2.1).

Table 2.1 Effect of caloric restriction on serum and tissue biochemistry of 6 month-old LCR rats. Male LCR (n>8 per group; 3 months of age) were fed ad libitum (AL) or caloric-restricted (CR) for 3 months prior to blood drawing from tail vein and tissue extraction at the time of sacrifice for biochemical analysis. Age-matched (6 month-old) AL-fed HCR were also examined. Values are expressed as mean ± SEM. A P < 0.05 AL-HCR vs AL-LCR; B P < 0.05 AL-HCR vs CR-LCR; C P < 0.05 AL-HCR vs CR-LCR; D P < 0.05 CR-LCR vs AL-LCR.

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<tr>
<td></td>
<td>AL</td>
<td>CR</td>
<td>AL</td>
</tr>
<tr>
<td>Initial Body Weight (g)</td>
<td>402. ± 12.6</td>
<td>412. ± 9.61</td>
<td>274. ± 8.41 A,C</td>
</tr>
<tr>
<td>Body Weight at sacrifice (g)</td>
<td>471. ± 8.29</td>
<td>335. ± 6.99 B</td>
<td>340. ± 9.81 A</td>
</tr>
<tr>
<td>Daily Food Consumption (g)</td>
<td>21.5 ± 0.52</td>
<td>12.3 ± 0.45 B</td>
<td>19.3 ± 0.55 C</td>
</tr>
<tr>
<td>% Visceral Fat/Body Weight</td>
<td>1.81 ± 0.11</td>
<td>0.81 ± 0.12 B</td>
<td>1.11 ± 0.11 A</td>
</tr>
<tr>
<td>Hepatic Triglyceride (μg/mg protein)</td>
<td>154. ± 20.5</td>
<td>67.8 ± 14.4 B</td>
<td>96.7 ± 13.8 A</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mg/dL)</td>
<td>121. ± 6.14</td>
<td>106. ± 3.38 B</td>
<td>103. ± 4.54 A</td>
</tr>
<tr>
<td>Random Blood Glucose (mg/dL)</td>
<td>100. ± 3.10</td>
<td>86.8 ± 4.70 B</td>
<td>87.3 ± 3.26 A</td>
</tr>
<tr>
<td>Serum Insulin (pM)</td>
<td>794. ± 156.</td>
<td>115. ± 12.2 B</td>
<td>201. ± 31.8 A,C</td>
</tr>
<tr>
<td>Serum C-peptide (pM)</td>
<td>2655 ± 259.0</td>
<td>940. ± 133. B</td>
<td>1397 ± 145.0 A,C</td>
</tr>
<tr>
<td>Serum C-peptide/Insulin Molar Ratio</td>
<td>4.66 ± 0.84</td>
<td>8.23 ± 0.62 B</td>
<td>7.81 ± 0.63 A</td>
</tr>
<tr>
<td>Serum FFA (mEq/L)</td>
<td>1.46 ± 0.11</td>
<td>0.70 ± 0.20 B</td>
<td>0.79 ± 0.07 A</td>
</tr>
<tr>
<td>Serum triglyceride (mg/dL)</td>
<td>53.1 ± 7.32</td>
<td>18.7 ± 3.05 B</td>
<td>31.9 ± 3.96 A,C</td>
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FIG. 2.1: Caloric restriction reduces body mass in LCR. LCR rats were randomized to either caloric restriction (CR) or ad libitum (AL) feeding (n=10 in each feeding group). Total body weight was measured over the course of the study and compared to that of AL-HCR (n=10). Values are mean ± SEM. AL-HCR and AL-LCR were significantly different (P < 0.001) at all feeding time points.

Caloric restriction improved hepatic insulin clearance and overall insulin sensitivity in LCR: By comparison to AL-HCR, AL-LCR exhibited hyperinsulinemia with impaired insulin clearance, as assessed by a ~2-fold lower steady state C-peptide-to-insulin molar ratio (Table 2.1). Caloric restriction reduced serum insulin level in LCR by ~7-fold. This could be due to its cumulative reducing effect on insulin secretion, as suggested by the 3-fold decrease in C-peptide levels (Table 2.1, CR- versus AL-LCR), and inducing effect on insulin clearance, as assessed by a 2-fold increase in C-peptide/Insulin ratio (Table 2.1, CR- versus AL-LCR). Consistent with a role for CEACAM1 in hepatic insulin clearance (DeAngelis et al., 2008a), Western analysis using a rat specific α-CC1 antibody for immunoblotting (Ib) followed by reprobing (reIb) with α-actin antibody, revealed >50% reduction in CEACAM1 level in AL-LCR by
comparison to AL-HCR livers (Figure 2.2A). Caloric restriction increased hepatic CEACAM1 protein levels to the level of AL-HCR (Figure 2.2A).

**Western analysis of liver proteins**

![Western blot analysis](image)

**FIG. 2.2:** The effect of caloric restriction on proteins implicated in lipid and insulin metabolism in liver of LCR. (A) Western analysis was performed in liver lysates by immunoblotting (Ib) with α-FAS, α-CEACAM1 (α-CC1) or α-CD36 antibodies followed by re-immunoprobing (Relb) with α-actin or α-tubulin to account for protein loading, as described in Materials and Methods. Two representative animals of at least 3 rats per each group are included. The mean of band density was measured and plotted as ± SEM in the graphs on the right. *P < 0.05 versus AL-LCR.

In addition to hyperinsulinemia, AL-LCR exhibited other features of insulin resistance by comparison to AL-HCR. These include glucose and insulin intolerance (Figure 2.3A and 2.3B, respectively), and fed hyperglycemia (Table 2.1), in agreement with other reports (Morris et al., 2009). Relative to AL-HCR, caloric restriction fully reversed glucose and insulin tolerance (Figure 2.3), indicating restoration of whole-body
insulin sensitivity. This suggests that caloric restriction ameliorated peripheral insulin action in parallel to elevating CEACAM1 levels in LCR livers.

**A. Glucose Tolerance Test**  

**B. Insulin Tolerance Test**

![Graphs showing glucose and insulin tolerance tests](image)

**FIG. 2.3:** Caloric restriction improves insulin and glucose tolerance in LCR. Rats (n=10 from each feeding group per line) were anesthetized and subjected to intraperitoneal glucose (A) and insulin (B) tolerance tests (IGTT and ITT, respectively). Values are mean ± SEM. *P < 0.05 versus AL-LCR, as analyzed by student’s independent t-test.

Of note, caloric restriction exerts a similar effect on body weight and insulin clearance in 9 month-old males from generation 18 (not shown).

Caloric restriction improved lipid metabolism in LCR: Consistent with elevation in key hepatic lipogenic enzymes, such as FAS, under conditions of chronic hyperinsulinemia (Assimacopoulos-Jeannet et al., 1995; Deng et al., 2002; McCormick et al., 1979), Western analysis revealed a higher FAS protein level in AL-LCR than HCR livers (Figure 2.2A). This could contribute to the 2-fold higher hepatic triglyceride content in AL-LCR, which may in part underlie the ~2.5-fold higher level of serum triglyceride in these rats (Table 2.1). Increased visceral obesity in AL-LCR (by 2-fold) could, at least in part, result from redistribution of triglyceride into WAT. Elevation in visceral obesity
might result in excessive lipolysis and hepatic fatty acid transport, as suggested by the 2-fold higher fasting serum free fatty acids (FFA) levels (Table 2.1) and hepatic CD36 protein content (Figure 2.2A) in AL-LCR when compared to AL-HCR.

Caloric restriction in LCR lowered hepatic FAS protein (Figure 2A) and triglyceride content (Table 2.1), and serum triglyceride level (Table 2.1). It also reduced visceral adiposity and consequently, serum FFA levels, rendering them all comparable to AL-HCR levels (Table 2.1). Consistent with the role of CD36 in fatty acid transport in liver, hepatic CD36 protein content was also reduced to the level of AL-HCR (Figure 2.2A).

Caloric restriction reduced NASH pathogenesis in LCR: LCR exhibit features of NASH, a more progressive form of steatosis (Abdeen et al., 2006; McClain et al., 2004). These include inflammation, steatosis and fibrosis. Accordingly, H&E staining of liver sections revealed scattered mononuclear inflammatory cell infiltration in AL-LCR (Figure 2.4A, panel 1, black arrows) and altered hepatocellular architecture with microsteatosis (Figure 2.4A, panel 1, brown arrows) and macrosteatosis (Figure 2.4A, panel 1, green arrows). Caloric restriction markedly reduced inflammation, as indicated by absence of inflammatory cell islands and normal hepatic architecture in CR-LCR (Figure 2.4A, panel 2), as in AL-HCR (Figure 2.4A, panel 3). Early fibrosis was also apparent in AL-LCR with a NASH-like "chicken-wire" pattern of Sirius red stain (Figure 2.4B, panel 1). These chicken-wire fibrogenic changes were not detected in either CR-LCR (Figure 2.4B, panel 2) or AL-HCR (Figure 2.4B, panel 3).
FIG. 2.4: Caloric restriction reduces features of NASH in LCR. (A) Liver sections (magnification, X40) from >5 rats of each of feeding group were analyzed by H&E staining, as described in materials and methods. Small (brown arrow) and large (green arrow) lipid-containing vacuolated cells were detected throughout the section from AL-LCR (Panel 1). Inflammatory cells foci were also noted in this animal (Panel 1, black arrows). (B) Sirius red staining revealed fibrotic changes in AL-LCR (Panel 1), but not in other animals. (C) TBARS assay was performed in liver lysates from 6 animals in each feeding group. Bars shown are mean ± SEM. *P < 0.05 versus AL-LCR, as analyzed by student’s independent t-test.
In agreement with increased oxidative stress in LCR (Thyfault et al., 2009), lipid peroxidation (assessed by TBARS concentration) was elevated in AL-LCR liver as compared to AL-HCR (Figure 2.4C). Caloric restriction reduced markedly TBARS concentration in LCR, rendering it comparable to that in AL-HCR (Figure 2.4C). This suggests that caloric restriction limited the initiation of oxidative changes in LCR liver.

Metabolic effect of endurance training in LCR liver: In previous studies, we have shown that eight weeks of high-intensity endurance training improved most of the cardiovascular abnormalities associated with the metabolic syndrome in LCR, glucose tolerance and insulin receptor phosphorylation and signaling in insulin target tissues (skeletal muscle, adipose tissue and liver) (Haram et al., 2009). In addition to reducing body weight (Haram et al., 2009), it also reduced visceral adiposity to the level of HCR (Table 2.2). However, this training program did not fully reverse hepatic and serum triglyceride levels, as these parameters remained higher in T-LCR than AL-HCR (Table 2.2). Sustained elevation in hepatic triglyceride level was due, at least in part, to persistent elevation in synthesis, as suggested by high levels of hepatic FAS protein content in T-LCR (Figure 2.5A). This appears to stem from failure of high-intensity training to reverse hyperinsulinemia and impaired insulin clearance (Table 2.2, C-peptide/Insulin ratio). Consistently, high-intensity training did not induce hepatic CEACAM1 protein content (Figure 2.5A) or insulin-stimulated phosphorylation (Figure 2.5B) sufficiently enough to reach the level in S-HCR, as shown by >50% lower phosphorylated CEACAM1 in the α-phosphotyrosyl immunopellet (α-pY). Moreover, endurance training did not reverse the elevation in lipid peroxidation, as indicated by
persistently high hepatic TBARS concentration in T-LCR by comparison to S-HCR (Figure 2.5C).

Table 2.2: Effect of endurance training on serum and tissue biochemistry of 6 month-old LCR rats. Male LCR (n=8 per group; 4 months of age) were left sedentary (S) or trained (T) for 2 months prior to blood drawing from tail vein and tissue extraction at the time of sacrifice for biochemical analysis. Age-matched (6 month-old) sedentary HCR were also examined. Values are expressed as mean ± SEM. \( ^A \) P < 0.05 AL-HCR vs AL-LCR; \( ^C \) P < 0.05 AL-HCR vs CR-LCR; \( ^B \) P < 0.05 CR-LCR vs AL-LCR.

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<td></td>
<td>S</td>
<td>T</td>
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<tr>
<td>%Visceral Fat/Body weight</td>
<td>5.1 ± 0.3</td>
<td>1.9 ± 0.3 ( ^B )</td>
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<tr>
<td>Hepatic Triglyceride (μg/mg protein)</td>
<td>62.0 ± 6.01</td>
<td>58.7 ± 6.85</td>
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<tr>
<td>Serum Insulin (pM)</td>
<td>493. ± 98.9</td>
<td>658. ± 190.</td>
</tr>
<tr>
<td>Serum C-peptide (pM)</td>
<td>2385 ± 550.8</td>
<td>3433 ± 795.6</td>
</tr>
<tr>
<td>Serum C-peptide/Insulin Molar Ratio</td>
<td>4.02 ± 0.57</td>
<td>4.60 ± 0.67</td>
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<tr>
<td>Serum FFA (mEq/L)</td>
<td>1.44 ± 0.17</td>
<td>0.52 ± 0.08 ( ^B )</td>
</tr>
<tr>
<td>Serum Triglyceride (mg/dL)</td>
<td>40.3 ± 3.13</td>
<td>34.2 ± 4.17 ( ^B )</td>
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FIG. 2.5: The effect of endurance training in LCR liver. (A) Liver lysates from rats, which had been subjected to aerobic interval endurance training (T), or left sedentary (S) (Haram et al., 2009), were analyzed by immunoblotting (Ib) with α-CC1 and α-FAS antibodies, followed by re-immunoprobing (Relb) with α-actin to normalize per protein loading. Two representative animals of at least 3 rats per each group are included. The mean of band density was measured and plotted as ± SEM in the graphs on the right. *P < 0.05 versus AL-LCR. (B) Liver lysates were treated with (+) or without insulin (−), immunoprecipitated (Ip) with α-phosphotyrosine antibody (α-pY) and analyzed by immunoblotting (Ib) with an antibody against phospho-CEACAM1 (α-pCC1). Gels are representative of three separate experiments performed on lysates from different mice per group per experiment. (C) TBARS assay was performed in liver lysates from 6 animals in each group. Bars shown are mean ± SEM. *P < 0.05 versus S-LCR, as analyzed by student’s independent t-test.
2.5 Discussion

We have previously shown that LCR exhibit the metabolic syndrome, including hyperinsulinemia, hypertriglyceridemia, visceral obesity, and body weight gain. Moreover, hyperinsulinemia in LCR stems from compromised insulin clearance, which is in turn, associated with reduction in hepatic CEACAM1 mRNA levels as compared to HCR (Wisloff et al., 2005). The current study shows that caloric restriction fully reverses the altered hepatic metabolic state in LCR, and that this implicates the recovery of hepatic CEACAM1 and its ensuing regulatory effect on insulin extraction. In contrast, endurance training fails to fully restore hepatic CEACAM1 content, and serum insulin and triglyceride levels.

Whether insulin resistance precedes or follows obesity in type 2 diabetes is unresolved (Srinivasan et al., 2003). However, LCR develop hyperinsulinemia and hypertriglyceridemia at 5 weeks of age, before visceral obesity and body weight increase (Wisloff et al., 2005), demonstrating a key role for altered insulin and triglyceride homeostasis in the abnormal metabolic state in this model. The marked reduction of hepatic CEACAM1 in LCR and the similarity in the phenotype to that in Ceacam1 mutants (L-SACC1 and Cc1\(^{-/-}\) mice) (DeAngelis et al., 2008a; Park et al., 2006b; Poy et al., 2002b) provide circumstantial evidence that altered CEACAM1-dependent insulin clearance pathways link the pathogenesis of hyperinsulinemia to liver steatosis, hypertriglyceridemia and visceral obesity in LCR.

The role of CEACAM1 in this process is further supported by the observation that caloric restriction, which induces hepatic CEACAM1 expression, is more effective in regulating insulin and lipid metabolism in this model than endurance training, which
exerts a milder effect on hepatic CEACAM1 expression. In this regard, endurance training does not fully reduce hepatic and serum triglyceride levels, in parallel to persistently elevated hepatic FAS protein content, which most likely results from chronically elevated levels of insulin in trained LCR. That training does not restore insulin and lipid metabolism is in agreement with other reports showing that it fails to reduce hyperinsulinemia and hepatic FAS mRNA levels in obese Zucker rats, in spite of decreasing body weight and total fat mass (Fiebig et al., 2002). The reversal effect of exercise on lipid metabolism and insulin action in skeletal muscle and WAT of LCR (Haram et al., 2009) precludes the possibility that any positive effect of this program on liver was diminished in the 48 hours after the last bout of exercise before sacrifice. This provides evidence that endurance program indeed exerts a preferential role on metabolism in skeletal muscle, as opposed to caloric restriction, which primarily affects insulin action and metabolism in liver.

Our observations of a significant lowering effect of caloric restriction in comparison to exercise on hepatic steatosis are supported by human studies indicating that caloric restriction alone, without exercise, reduces liver triglyceride in a small cohort of obese individuals (Larson-Meyer et al., 2008). While these studies did not subject a group of obese humans to an exercise program alone, they serve to emphasize the beneficial therapeutic role of caloric restriction on lipid metabolism in liver.

The mechanism underlying the regulatory effect of caloric restriction on CEACAM1 expression is not well delineated. However, we have shown an inverse effect of high-fat intake and mobilization of fatty acids from WAT during fasting on CEACAM1 transcription (manuscript under consideration). The negative effect of
CEACAM1 on FAS enzymatic activity (Najjar et al., 2005a) implicates it in the regulation of the level of malonyl-CoA, a key determinant of fatty acid transport to mitochondria for β-oxidation. Thus, it is likely that under conditions of caloric restriction, CEACAM1 is elevated to reduce insulin levels in LCR, and consequently, hepatic FAS content. Together with inhibiting FAS enzymatic activity, this elevates malonyl-CoA levels to reduce β-oxidation, and ultimately, gluconeogenesis. This notion is supported by inactivation of the peroxisome proliferator-activated receptor α (PPARα), as suggested by reduced hepatic levels of one of its targets, CD36, and the reduction of fasting blood levels by caloric restriction.

As recently reported (Thyfault et al., 2009), LCR exhibit features of NASH, including steatohepatitis, fibrosis and elevation in oxidative stress. In agreement with other reports showing improvement of hepatic histology and reversal of hepatic steatosis and insulin resistance with a >9% loss of body weight by dietary restriction in humans (Harrison et al., 2009), caloric restriction reverses most, if not all NASH-characteristic features of LCR. Given the cumulative effect of increased fat deposition in liver and macrophage infiltration from WAT on the hepatic inflammatory milieu (Bigorgne et al., 2008a; Sheth et al., 1997), it is not all that surprising that LCR develop steatohepatitis. CEACAM1 also acts as a co-inhibitory receptor after T cell activation to inhibit inflammation (Gray-Owen and Blumberg, 2006). Thus, it is likely that reduction in hepatic CEACAM1 levels predisposes LCR to develop a more progressive form of hepatic steatosis. Consistent with the partial dependence of the anti-inflammatory function on the phosphorylation of immunoreceptor tyrosine-based inhibition motifs within the cytoplasmic domain of CEACAM1 (Nagaishi et al., 2006), L-SACC1 mice
exhibit key features of NASH when exposed to conditions that trigger inflammation, such as high-fat feeding (Lee et al., 2008). The development of NASH in LCR as in L-SACC1 mice, and conversely, the complete reversal of the NASH-characteristic phenotype by caloric restriction in parallel to inducing hepatic CEACAM1 levels lend further support to the role of CEACAM1-dependent pathways in the pathogenesis of the altered metabolic state in LCR.

In summary, the current studies demonstrate that although exercise alone improves insulin action in muscle and WAT, and reduces body weight in LCR (Haram et al., 2009), caloric restriction primarily targets insulin and lipid metabolism in liver. Although a cause-effect relationship is not well delineated, our results point to a differential inducing effect on hepatic CEACAM1 levels by caloric restriction as compared to exercise. Further studies are required to identify the CEACAM1-dependent pathways that underlie the beneficial effect of caloric restriction on hepatic metabolism. However, using this unique animal model of metabolic syndrome, this study provides suggestive evidence that CEACAM1 constitutes an important pharmacological target to treat this disease.

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Chapter 3

CEACAM1 Repression by PPARalpha as an Obligate Step in Dietary Insulin Resistance

Qusai Y. Al-Share\textsuperscript{1,2}\*, Thomas A. Bowman\textsuperscript{1,2}\*, Anthony M. DeAngelis\textsuperscript{1,2}, Sadeesh K. Ramakrishnan\textsuperscript{1,2}, Sumona Ghosh\textsuperscript{1,2}, Payal R. Patel\textsuperscript{1,2}, Garrett Heinrich\textsuperscript{1,2}, Sang Jun Lee\textsuperscript{1,2}, John J. Lazarus\textsuperscript{1,3}, Jiang Hu\textsuperscript{4}, R. Mark Wooten\textsuperscript{1,3}, Stanislaw M. Stepkowski\textsuperscript{1,3}, Anthony W. Ferrante Jr.\textsuperscript{5}, Frank J. Gonzalez\textsuperscript{6}, William Philbrick\textsuperscript{7}, Rohit N. Kulkarni\textsuperscript{4}, Yatrik M. Shah\textsuperscript{8} and Sonia M. Najjar\textsuperscript{1,2,†}

\textsuperscript{1} Center for Diabetes and Endocrine Research and Departments of \textsuperscript{2} Physiology and Pharmacology and \textsuperscript{3} Medical Microbiology and Immunology at the University of Toledo College of Medicine, Health Science Campus, Toledo, OH 43614; \textsuperscript{4} Joslin Diabetes Center, Harvard Medical School, Boston, MA 02215; \textsuperscript{5} Naomi Berrie Diabetes Center, Columbia University, New York, NY 10032; \textsuperscript{6} Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, \textsuperscript{7} Department of Internal Medicine, Section of Endocrinology and Metabolism, Yale University School of Medicine, New Haven, CT 06520, and \textsuperscript{8} Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, 48109

\*Authors contributed equally to these studies

†Address correspondence to:
Sonia M. Najjar, Ph.D.
College of Medicine
University of Toledo, Health Science Campus
3000 Arlington Avenue, Mail stop 1008
Toledo, Ohio, 43614
Tel: (419) 383-4183
FAX: (419) 383-2871
e-mail: sonia.najjar@utoledo.edu

Running Title: CEACAM1 in diet-induced insulin resistance
Nonstandard abbreviations used. CEACAM1, CarcinoEmbryonic Antigen-related Cell Adhesion Molecule 1; Ceacam1, gene encoding CEACAM1 protein; Cc1−/−, global Ceacam1 null mouse; L-SACC1, liver-specific S503A CEACAM1 mutant mouse; BL6, C57BL/6 genetic background; PPARα peroxisome proliferator-activated receptor α; PPARα+/− null mutant PPARα mouse; Wy, Wy14,643 PPARα agonist; RD, regular diet; HF, high fat diet; FFA, free fatty acids; FATP-1, fatty acid transport protein-1; FAS, fatty acid synthase.

Conflict of interest: The authors have declared that no conflict of interest exists.
3.1 Abstract

OBJECTIVE—Impaired hepatic insulin clearance causes hyperinsulinemia and secondary insulin resistance. It is unclear whether hyperinsulinemia contributes to worsening insulin resistance by down-regulating insulin receptors and promoting hepatic lipid accumulation.

RESEARCH DESIGN AND METHODS—Mice on C57BL/6 genetic background were fed ad libitum an equicaloric diet of either a standard chow with low 12% fat content (RD) or 45% high-fat diet (HF) for 30 days before their metabolic phenotype was characterized. Additionally, a transgenic mouse with liver-specific overexpression of rat CEACAM1 (L-CC1) was generated to evaluate the role of hepatic CEACAM1 in diet-induced insulin resistance and metabolic abnormalities.

RESULTS—We show that an early event associated with high fat feeding is repression of CEACAM1 by a PPARα-mediated mechanism, and that this leads to impaired insulin clearance prior to the development of a pro-inflammatory state. Transgenic over-expression of CEACAM1 in liver prevents hyperinsulinemia and insulin resistance, and limits visceral obesity and the metabolic response to high-fat intake.

CONCLUSIONS—We propose that hyperinsulinemia resulting from impaired insulin clearance is an early cause, and not a consequence of diet-induced insulin resistance, and that the latter can be reversed by restoration of hepatic CEACAM1 level.

Keywords: CEACAM1/insulin resistance/high-fat diet/insulin clearance/hyperinsulinemia/PPARα
3.2 Introduction

Insulin resistance is a key factor in the etiology of metabolic diseases, and is commonly heralded by hyperinsulinemia. Although hyperinsulinemia reflects impaired response in insulin target tissues, the cause-effect relationship between hyperinsulinemia and insulin resistance is complex. Peripheral insulin resistance causes secondary compensatory hyperinsulinemia, resulting from increased insulin secretion from β-cells (Polonsky et al., 1988; Valera Mora et al., 2003). But hyperinsulinemia, caused by impaired insulin clearance, worsens insulin resistance by down-regulating insulin receptors and escalating de novo lipogenesis.

Considerable evidence in humans supports the view that impaired hepatic insulin extraction causes chronic hyperinsulinemia in obesity (Escobar et al., 1999; Polonsky et al., 1988). Moreover, modest weight loss (10%) is associated with a greater improvement of insulin clearance than insulin secretion, which requires a more marked weight loss (Jones et al., 2000). Studies on the role of the carcinoembryonic-related cell adhesion molecule 1 (CEACAM1) in insulin clearance provide more convincing evidence that hyperinsulinemia causes insulin resistance (Poy et al., 2002b). Mice with liver-specific Ceacam1 inactivation (L-SACC1) or with global null mutation (Cc1−/−), exhibit impairment of insulin clearance and hyperinsulinemia, which causes insulin resistance (DeAngelis et al., 2008b; Park et al., 2006a). These mice are also viscerally obese, in part due to redistribution of triglyceride from liver to white adipose tissue (WAT).

Ceacam1 mutant mice demonstrate that impaired insulin clearance causes insulin resistance and visceral obesity. Reciprocally, increased visceral obesity causes insulin
resistance by activating FFA- and adipokines-mediated pathways (Lazar, 2005; Sjoholm and Nystrom, 2005; Tataranni and Ortega, 2005). Increased FFA supply causes insulin resistance in liver, at least in part, by impairing insulin clearance through mechanisms involving activation of PKC-delta (Chen et al., 2006) and reducing insulin receptor number (Svedberg et al., 1990). FFA supply also contributes to lipid accumulation in hepatocytes, which in turn induces local inflammatory response (Bigorgne et al., 2008b) and elevates TNFα production (Boden, 2006) to inactivate insulin signaling (Cai et al., 2005; Hotamisligil et al., 1993). Similarly, a rise in WAT-derived adipokines modulates the intra-hepatic pro-inflammatory milieu (de Wit et al., 2008; Li et al., 2005) to contribute to pathways leading to insulin resistance.

The most common form of visceral obesity is diet-induced. Prolonged fat intake causes visceral obesity and insulin resistance, which has been explained in part with the development of a chronic inflammatory state (Chiang et al., 2009; Shi et al., 2006). However, as opposed to other cardinal features of obesity (hyperinsulinemia, insulin resistance, dysplidemia), inflammation (characterized by elevation in WAT-associated and circulating adipokines) constitutes a delayed component of the progression of the disease, and in response to high-fat diet, it emanates from a sustained period of nutrient overload (>3 months) (Kim et al., 2008). Based on previous work underscoring the central role for CEACAM1 in pathways regulating insulin metabolism and de novo lipogenesis (Najjar et al., 2005b), we investigated whether CEACAM1 is implicated in the early pathogenesis of diet-induced insulin resistance. We herein, show that hyperinsulinemia causes insulin resistance, and is not simply a consequence thereof.
3.3 Research Designs and Methods

Animal husbandry. Male mice were kept in a 12h-dark/light cycle. All procedures were approved by the Institutional Animal Care and Utilization Committee.

Wild-type mice on the C57BL/6 (BL6) background were from Jackson Laboratories, and BL6.PPARα−/− and PPARα+/+ mice from Taconic laboratories.

Like the L-SACC1 mouse (Poy et al., 2002b), L-CC1 transgenic mice with liver-specific over-expression of rat CEACAM1 were generated using the proximal 490 bp-fragment 5’ of the translation start site in the human apolipoprotein (Apo) A-I promoter/enhancer element. The ApoAI/WT Ceacam1 minigene, containing intron 1, the BGH polyadenylation signal and a Flag-tag at the 3’ end, was excised and injected in the pronuclei of single-cell fertilized mouse embryos from SJLXC57Bl/6J matings (Yale Transgenic Facility). 11 F0 founders were identified by PCR analysis of tail gDNA and liver-specific transgene expression was confirmed by Western analysis using the rat-specific CEACAM1 polyclonal antibody (Poy et al., 2002b) and a monoclonal anti-FLAG M2 antibody (SIGMA cat# F1804). Two lines were identified and backcrossed onto FVB background and then twice onto BL6 (~75% BL6) for 30 days of high-fat feeding. Mice were further backcrossed into both BL6 and FVB lines. Extended high-fat feeding was with mice on the FVB genetic background in order to better observe the potential beneficial effects that liver may have on muscle and adipose tissue.

Male mice (2-3 months of age) were fed ad libitum either a standard chow (Teklad 2016) with low 12% fat content (RD) or a 45% high fat (HF) diet (Research Diets, Catalog#
In some experiments, mice were fed a chow mixed with 0.1% w/w of Wy14,643 PPARα agonist (Biomol International) for 3 days.

Analysis of metabolic parameters. Following an overnight fast, mice were anesthetized at 1100h. Whole venous blood was drawn to measure the levels of glucose, serum insulin, C-peptide, free fatty acids (FFA) and triglycerides, as described previously (DeAngelis et al., 2008b). Serum adipokine levels were determined using the LincoPlex Mouse Adipokine Kit following a 6 hour-fast (Weisberg et al., 2006). Hepatic triglyceride and glycogen content was measured as described previously (Park et al., 2006a). Visceral adipose tissue was excised, weighed, and visceral adiposity expressed as percentage of body weight (BWt).

Insulin and glucose tolerance tests. Following an overnight fast, mice were anesthetized, injected intraperitoneally with Regular Human insulin (Novo Nordisk, 0.125U/kg BWt) or glucose (1.5 or 2.0g/kg body weight) before drawing blood for glucose measurement (DeAngelis et al., 2008b).

Western analysis. Proteins were analyzed by SDS-PAGE (Invitrogen) and immunoprophbing with polyclonal antibodies against endogenous mouse CEACAM1 (α-CC1) (Najjar et al., 2005b), rat CEACAM1 (α-rCC1) (DeAngelis et al., 2008b), fatty acid synthase (α-FAS) (Najjar et al., 2005b), PPARα (Santa Cruz), CD36 (Santa Cruz) and FATP-1 (Santa Cruz). Monoclonal antibodies against PPARγ (Santa Cruz) and Flag (M2, Sigma) were also used. For normalization, membranes were reprobed with monoclonal antibodies against Actin, GAPDH and Tubulin (Sigma or Santa Cruz). Proteins were detected by enhanced chemiluminescence (ECL; Amersham).
Ex-vivo phosphorylation. Tissue lysates were treated with 100nM insulin for 5min prior to immunoprecipitation with antibodies against the β-subunit of the insulin receptor (α-IRβ, Santa Cruz Biotechnology) or phospho-Akt (Ser\textsuperscript{473}, Cell Signaling Technology), SDS-PAGE analysis and immunoblotting with α-phosphotyrosine (α-pY) antibody (Upstate Biotechnology Inc). Membranes were reprobed with α-IRβ and α-Akt (Cell Signaling Technology) for normalization.

Northern blot. Liver and cellular mRNA was purified using Trizol (Invitrogen) followed by the MicroPoly (A) Pure kit (Ambion) and analysis by probing with cDNAs for peroxisome proliferator activated receptor-α (ppara) and Ceacam1, using the Random Primed DNA Labeling Kit (Roche), prior to reprobing with β-actin cDNA to normalize against the amount of mRNA applied.

Cell culture. FAO rat hepatoma and human embryonic kidney cells (HEK293) were maintained at 5%CO\textsubscript{2} in Dulbecco-Modified Eagle's Medium (DMEM) (Cellgro). At ~80% confluency, cells were grown in media with 10% dialyzed fetal bovine serum-FBS (Invitrogen) for 16h before incubating in ethanol (vehicle) or 100μM Wy14,643 (Biomol) followed by Western and Northern analyses.

Luciferase assay. Using Lipofectamine 2000 (Invitrogen), H4-II-E rat hepatoma and HEK293 cells, grown in DMEM, were co-transfected with 300ng of pGL3 containing the rat ceacam1 promoter (−1609/−21) or its 5'-deletion constructs (Najjar et al., 1996), and 30ng of renilla luciferase (pRL-TK, Promega). Following treatment with vehicle (ethanol) or with Wy14,643 for 24h, cells were lysed, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).
Chromatin immunoprecipitation (ChIP). As described (Kamiya et al., 2004), mice were fed a Wy14,643-supplemented diet for 2 weeks prior to liver extraction, grinding under liquid nitrogen and crosslinking in 1% formaldehyde-1XPBS at 37°C for 20min. Crosslinking was terminated with 125mM glycine and the cell pellet washed with 1XPBS. Nuclei were lysed in SDS buffer (50mM Tris-HCl pH 8.1, 10mM EDTA, 1% SDS and Protease inhibitors), chromatin sheared by sonication and the nuclei lysate cleared by centrifugation at 50,000xg for 30 min. The soluble chromatin was diluted 10-fold in (0.01% SDS, 1.1% TritonX100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, 167mM NaCl), and immunoprecipitated with α-PPARα antibody (Geneka or Santa Cruz Biotechnology; H-98). The antibody/protein/DNA complex was isolated using magnetic beads conjugated with protein A (New England Biolabs). The protein/DNA complex was washed, eluted in 50mM NaHCO₃-1% SDS, and left at 65°C overnight to reverse crosslinking. Following protein digestion with proteinase K for 1h at 45°C, DNA was purified by phenol/chloroform/isoamyl alcohol extraction, and PCR-amplified using a proximal (forward 5'-TTTCCAGGACACACAGGTCACC-3'; reverse: 5'-GCAGCAGGAGTTCCAGCATTTC-3') and a distal primer set (forward 5'-GCTTCATTCCACTCTCTCCTCC-3'; reverse 5'-CATACAGCCCCAGCAGGTCTTTG-3'), which yields a 270 bp-product each. For the malic enzyme control, the primer set yielding a 190 bp was used (forward 5'-TCTTGGTTTCCAGAACGCTCA-3' and reverse: 5'-TCGTCCTACTGTCCTCCCATAC-3').

For quantitative ChIP analysis of PPARα binding to Ceacam1 promoter in mice fed a HF diet, DNA fragments were subjected to real-time qPCR (Step One Plus, Applied Biosystems) using primers flanking PPRE on the ceacam1 promoter (Forward: 5'-
CAGGGCACAGAGGGCTCCCA-3' and Reverse: 5'-GCGGGCCTCCCGTCCTTCTA-3'). Fold enrichment of PPARα binding to ceacam1 promoter region was calculated by normalizing against input and control with no antibody.

Semi-quantitative Real-Time RT-PCR. Total hepatic RNA was isolated with PerfectPure RNA Tissue Kit (5 Prime) and total adipose tissue RNA was isolated with RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized by ImProm-II™ Reverse Transcriptase (Promega), using 1μg of total RNA and primers (Table S1). cDNA was evaluated with real-time quantitative PCR (Step One Plus, Applied Biosystems). The relative amounts of mRNA were calculated by comparison to the corresponding standards and normalized relative to β-actin. Results are expressed in fold change as mean ± SEM.

Statistical analysis. Data were analyzed with SPSS software using one-factor ANOVA analysis or Student’s t-test and graphed with GraphPad Prism 4 software. P<0.05 were statistically significant.
3.4 Results

High fat diet reduces hepatic CEACAM1 levels and impairs insulin clearance. High-fat (HF) feeding for 9-30 days increases visceral obesity in male BL6 mice (Table 3.1). This effect is reversed upon switching to a regular diet for the next 30 days (30/30RD) (Table 3.1).

Table 3.1: Effect of high-fat diet on serum and tissue biochemistry in C57BL/6 male mice. Male mice (4 months of age, n>6 per group) were fed RD or HF for 9 and 30 days. Some were fed back RD for 30 days after 30 days of HF (30/30RD). * P < 0.05 HF versus RD. Values are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>9 Days</th>
<th>30 Days</th>
<th>30/30 Days</th>
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<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
<td>RD</td>
</tr>
<tr>
<td>% Visceral Fat/Body Wt.</td>
<td>1.2±0.1</td>
<td>1.9±0.1</td>
<td>1.4±0.3</td>
</tr>
<tr>
<td>Serum Insulin (pM)</td>
<td>362±7.2</td>
<td>399±10.4</td>
<td>356±16.6</td>
</tr>
<tr>
<td>Serum C-Peptide (pM)</td>
<td>266±34.0</td>
<td>277±22.0</td>
<td>307±53.2</td>
</tr>
<tr>
<td>C-Peptide/Insulin</td>
<td>0.8±0.2</td>
<td>0.7±0.1</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Hepatic Triglyceride (µg/mg protein)</td>
<td>183±23.8</td>
<td>312±67.6</td>
<td>166±21.1</td>
</tr>
<tr>
<td>Serum FFA (mEq/l)</td>
<td>0.5±0.1</td>
<td>0.7±0.1</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mg/dl)</td>
<td>100±2.10</td>
<td>104±5.60</td>
<td>96.5±5.90</td>
</tr>
<tr>
<td>Fed Blood Glucose (mg/dl)</td>
<td>NA</td>
<td>NA</td>
<td>103±4.41</td>
</tr>
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Northern and Western analyses indicate that CEACAM1 mRNA and protein levels are, respectively, reduced by > 50% after 30, but not 9 days of HF intake (Fig. 3.1A). Switching back to RD restores CEACAM1 levels (30/30RD) (Fig. 3.1A).
FIG 3.1. The effect of high-fat diet on insulin action and CEACAM1. (A) The effect of high-fat diet on hepatic CEACAM1 levels in BL6 mice. 4 month-old BL6 male mice were fed a regular chow (RD) or a high-fat diet (HF) for 9-30 days. After 30 days of HF intake, some were fed RD for the next 30 days (30/30RD, or in graph HF, RD). Liver lysates were subjected to Northern (i) and Western (ii) analyses with reprobing with β-actin cDNA and α-actin antibody, respectively, for normalization. Two out of > 6 mice from each feeding category per mouse group are shown. Bands were scanned from all gels, and the density measured with arbitrary units and represented in the graphs on the right hand-side. *P<0.05 vs RD. (B) Following the indicated days of feeding, mice (n>6 per feeding category per mouse group) were challenged with an intra-peritoneal glucose (2mg/kg body weight) or (C) insulin (0.75 U/kg body weight) injection to assess blood glucose levels at 0-120 or 0-180 min. post-injection. *P<0.05 HF (open squares) vs. RD (closed circles).
FIG 3.2 (A) Lysates from liver, soleus muscle and white adipose tissue (WAT) from mice fed HF or RD for 30 days were treated with buffer (–) or 100 µM insulin (+) in the presence of ATP. Insulin receptor (IRβ) was immunoprecipitated (Ip) with α-IRβ antibody, and analyzed by SDS PAGE and immunoblotting (Ib) with α-phosphotyrosine (α-pY) prior to reprobing with α-IRβ (reIb) for normalization. Separate aliquots of lysates were subjected to sequential immunoblotting initially with α-phospho-Akt followed by α-Akt antibody. Gels represent 3 mice per feeding category per mouse group. (B) Tissue lysates were subjected to Western analysis, which includes reprobing with α-actin, to determine protein content of fatty acid synthase (FAS) and CD36 in liver, and of FATP-1 in Soleus muscle and WAT. Gels represent 3 mice per feeding category per mouse group.

Consistent with a key role for CEACAM1 in hepatic insulin clearance, HF intake for 30, but not 9 days, decreases insulin clearance (as measured by steady state C-peptide/Insulin molar ratio-C/I) and causes hyperinsulinemia (P<0.05; Table 3.1). Switching back to RD reverses this effect on both insulin clearance and insulin (Table 3.1, 30/30 days; HF/RD as compared to RD/RD).

HF causes hepatic insulin resistance in a CEACAM1-dependent manner. Clamp analysis demonstrated that insulin resistance requires about 30 days of continuous fat intake to develop in BL6 mice (Park et al., 2005). Accordingly, we herein show that HF for 22 to
28, but not 12 to 15 days, causes glucose intolerance (Fig. 3.1B) and delayed glucose recovery following insulin injection during insulin tolerance test (Fig. 3.1C) and decreases phosphorylation of the insulin receptor β-subunit (IRβ) by insulin in liver lysates (Fig. 3.2A). Switching back to RD (30/30RD) restores glucose tolerance and insulin signaling in liver (not shown).

Consistent with the development of chronic hyperinsulinemia, hepatic FAS protein level is elevated in mice fed a HF for 30 days (Fig. 3.2B). Together with elevated CD36 protein levels (Fig. 3.2B), this suggests increased hepatic lipid transport and lipogenesis that contribute to increased hepatic triglyceride content after 30 days of HF intake (Table 3.1). Increased visceral obesity (Table 3.1) and elevated protein levels of FATP-1, a major fatty acid transporter, in white adipose tissue (WAT) (Fig. 3.2B), suggest redistribution of triglyceride to this tissue. Consistent with human studies (Bays et al., 2004), this is associated with increased lipolysis and elevation in plasma FFA levels (Table 3.1) without causing insulin resistance in WAT, as assessed by normal IRβ and Akt phosphorylation in response to insulin (Fig. 3.2A). Whereas HF fails to alter IRβ phosphorylation in soleus muscle (Fig. 3.2A), it reduces post-receptor insulin signaling through Akt. This proposes reduction in glucose uptake in muscle. The data suggest that HF intake for 30 days causes insulin resistance primarily in liver and to some extent in muscle, in addition to visceral obesity.

HF intake for 12-21 days reduces CEACAM1 mRNA and protein levels by ~35-50% without significantly affecting IRβ phosphorylation in response to insulin in liver (not shown). This gene-dosage effect indicates that HF causes hepatic insulin resistance via a mechanism requiring >50% loss in hepatic CEACAM1 level. This finding is consistent
with the normal metabolic phenotype of heterozygous Cc1<sup>−/−</sup> null mice (DeAngelis et al., 2008b).

HF intake for 30 days does not cause a robust inflammatory response. No significant changes are observed in mRNA levels of WAT-associated macrophage markers (CD68 and F<sub>4</sub>/80) and adipokines (IL-1β, IL-6, and TNF-α) by a 30 day-HF intake (Table 3.2). Except for leptin, which is elevated by ~5-fold in HF-fed mice, most serum adipokines tested, such as the Monocyte Chemoattractant Protein-1 (MCP-1), do not rise significantly (Table 3.2).

Table 3.2: Effect of high-fat diet on macrophage markers and adipokine levels in white adipose tissue (WAT) and serum of C57BL/6 male mice. Male mice (4 months of age; n=7-10 mice/group) were fed HF for 30 days. *P < 0.05 HF versus RD. Values are expressed as mean ± SEM. ND = Not Detected.

<table>
<thead>
<tr>
<th></th>
<th>RD</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAT CD68</td>
<td>0.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>F&lt;sub&gt;4&lt;/sub&gt;/80</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>IL-6</td>
<td>3.0 ± 0.9</td>
<td>2.9 ± 1.0</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Serum MCP-1 (pg/ml)</td>
<td>50 ± 9</td>
<td>59 ± 15</td>
</tr>
<tr>
<td>Serum TNF-α</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>585 ± 96</td>
<td>3373 ± 1354 &lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Liver-specific overexpression of CEACAM1 protects against insulin resistance. To investigate the cause-effect relationship between loss of CEACAM1 and diet-induced insulin resistance, we generated mice (termed L-CC1) overexpressing FLAG-tagged rat CEACAM1 in liver, using the ApoA1 promoter (Poy et al., 2002b). Immunoblotting (Ib)
liver lysates with α-FLAG and α-rat CEACAM1 (α-rCC1) antibodies shows that ApoA1 drives liver-specific expression, as assessed by absence of expression in intestine (not shown), the other main site of ApoA1 production (Rader, 2006).

FIG. 3.3 Liver-specific overexpression of CEACAM1 in liver protects against diet-induced insulin resistance. (A) Wild-type (WT) and L-CC1 mice with liver-specific overexpression of rat Ceacam1 were fed a HF or RD for 30 days. Livers were excised and their lysates analyzed by Western blotting using antibodies against rat (rCC1) and mouse (mCC1) proteins to determine the protein level of transgenic and endogenous CEACAM1, respectively. The bar graph represents semi-quantitative real-time qRT-PCR analysis of mRNA from these livers using primers common to both rat Ceacam1 and endogenous mouse Ceacam1, and normalized to Gapdh. *P<0.05 HF vs RD in the same mouse group and #P<0.05 vs WT-RD. (B) Following incubation with buffer (–) or 100µM insulin (+) in the presence of ATP, liver lysates were subjected to immunoprecipitation (Ip) with α-pTyr antibody, and analyzed by SDS-PAGE and immunoblotting (Ib) with an antibody against the insulin receptor (α-IRβ). Gels represent >3 mice per feeding category per mouse group. (C) Mice (n>6 per feeding group per mouse group) were fed RD or HF for 30 days prior to receiving an ip injection of glucose (1.5mg/kg body weight) or insulin (0.75 U/kg body weight) and their blood glucose level determined post-injection. *P<0.05 HF (closed squares-WT, or circles-L-CC1) vs RD (open squares-WT, or circles-L-CC1).

Unlike endogenous mouse CEACAM1, which undergoes a >55% (p<0.05) reduction after 30 days of HF intake when propagated on a FVBxBL6 background (Fig. 3.3A, Ib: α-mCC1), transgenic rat CEACAM1 protein level remains intact (Fig. 3.3A, Ib: α-rCC1), owing to ApoA1 induction by HF (not shown). Similarly, qRT-PCR analysis using
primers common to both rat and mouse Ceacam1 indicates that in contrast to the decrease in endogenous mouse Ceacam1 mRNA (Fig. 3.3A graph, grey vs. orange bar), total hepatic Ceacam1 mRNA level remains ~5.5-fold higher in L-CC1 than WT mice (Fig. 3.3A graph, green bars vs grey bar).

Table 3.3 Effect of 30 days of a high-fat intake on serum and tissue biochemistry of L-CC1 mice. Male mice (n>8) were fed a HF diet for 30 days starting at 2 months of age. *P<0.05 HF versus RD. Values are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>L-CC1</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>28. ± 0.5</td>
<td>34. ± 0.7 *</td>
</tr>
<tr>
<td>% Visceral Fat/Body Wt.</td>
<td>.67 ± .04</td>
<td>2.0 ± .49 *</td>
</tr>
<tr>
<td>Fasting Serum Insulin (pM)</td>
<td>53. ± 5.8</td>
<td>112. ± 19.0 *</td>
</tr>
<tr>
<td>Fasting Serum C-peptide (pM)</td>
<td>403. ± 84.0</td>
<td>568. ± 148.</td>
</tr>
<tr>
<td>Steady State C/I</td>
<td>7.6 ± 0.6</td>
<td>5.1 ± 0.6 *</td>
</tr>
<tr>
<td>Hepatic Triglyceride (µg/mg protein)</td>
<td>83. ± 7.0</td>
<td>122. ± 13.2 *</td>
</tr>
<tr>
<td>Fasting Serum Triglyceride (mg/ml)</td>
<td>61. ± 11.</td>
<td>105. ± 7.4 *</td>
</tr>
</tbody>
</table>

Although insulin levels in this mice set are lower than in BL6 mice (Table 3.1), due in part to the contribution of the FVB background (Berglund et al., 2008), HF feeding for 30 days causes hyperinsulinemia in WT, but not L-CC1 mice (Table 3.3). This appears to be due to the differential negative effect of HF on insulin clearance in WT mice (C/I -Table 3.3). As opposed to WT, HF does not increase body weight, visceral obesity, and hepatic and serum triglyceride content in L-CC1 mice (Table 3.3). Moreover, 30 day-fat feeding does not compromise insulin action in L-CC1 mice, as assessed by intact insulin.
induction of IR\(\beta\) phosphorylation in liver lysates (Fig. 3.3B) and glucose and insulin tolerance (Fig. 3.3C). This emphasizes that protection of hepatic CEACAM1 levels prevents metabolic abnormalities in response to 30 days of HF intake.

HF feeding for 6 months causes hyperinsulinemia in WT, but not L-CC1 mice (Table 4). This appears to be due to the differential negative effect of HF on insulin clearance in WT mice (C/I -Table 3.4). As opposed to WT, in L-CC1 mice 6 months of HF does not increase fasting blood glucose, increase visceral adiposity, and free fatty acids and muscle triglyceride content, despite an increase in body weight and hepatic triglyceride content (Table 3.4).

Table 3.4: Effect of 6 months of a high-fat intake on serum and tissue biochemistry of L-CC1 mice. Male mice (n>8) were fed a HF diet for 6 months starting at 2 months of age. *P<0.05 HF versus RD. #P<0.05 LCC1 versus WT of same diet. Values are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>L-CC1</th>
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<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td>% Visceral fat weight</td>
<td>2.26 ± 0.12</td>
<td>5.16 ± 0.50*</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>144. ± 11.6</td>
<td>191. ± 14.0*</td>
</tr>
<tr>
<td>Hepatic triglyceride (ng/µg protein)</td>
<td>13. ± 3</td>
<td>137. ± 24*</td>
</tr>
<tr>
<td>Muscle triglyceride (ng/ µg protein)</td>
<td>34. ± 5</td>
<td>138. ± 17*</td>
</tr>
<tr>
<td>Serum FFA (mEq/l)</td>
<td>0.62 ± 0.04</td>
<td>0.82 ± 0.06*</td>
</tr>
<tr>
<td>Serum insulin (pM)</td>
<td>77.8 ± 16.9</td>
<td>162. ± 9.2*</td>
</tr>
<tr>
<td>Serum C-peptide (pM)</td>
<td>987 ± 386</td>
<td>1092 ± 162</td>
</tr>
<tr>
<td>Serum C-peptide/insulin (M ratio)</td>
<td>12.7 ± 2.8</td>
<td>6.72 ± 1.4*</td>
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</table>
Liver-specific overexpression of CEACAM1 protects against long-term high-fat diet induced metabolic abnormalities. Similar to 30 days of HF intake, unlike endogenous mouse CEACAM1, which undergoes a >55% (p<0.05) reduction after 6 months of HF intake (Fig. 3.4A, lb: α-mCC1), transgenic rat CEACAM1 protein level remains intact (Fig. 3.4A, lb: α-rCC1). Real-time qRT-PCR analysis using primers common to both rat
and mouse Ceacam1 indicates that in contrast to the decrease in endogenous mouse Ceacam1 mRNA (Fig. 3.4A graph, grey vs orange bar), total hepatic Ceacam1 mRNA level remains ~5 to 6-fold higher in L-CC1 than WT mice (Fig. 3.4A graph, green bars vs. grey bar).

Moreover, up to 6 months of fat feeding does not compromise insulin action in L-CC1 mice, as assessed by intact insulin stimulated glucose uptake in isolated soleus muscle (Fig. 3.4B) and glucose tolerance (Fig. 3.4C). Further, insulin tolerance test displays faster recovery after insulin infusion, consistent with improved insulin clearance (Fig. 4D). These results emphasize that protection of hepatic CEACAM1 levels prevents metabolic abnormalities in response to up to 6 months of HF intake.

FIG. 3.5: L-CC1 mice are protected from features of NASH in 4 months HF. (A) Liver sections (magnification, X40) from >5 rats of each of feeding group were analyzed by H&E staining, as described in materials and methods. Small and large (arrows) lipid-containing vacuolated cells were detected throughout the section from WT-HF. Inflammatory cells foci were also noted in WT-HF (red circle). These changes were not present in LCC1-HF. (B) WAT sections (magnification X40) from >5 rats of each feeding group were analyzed by H&E staining. Very large lipid-containing vacuolated cells were detected throughout the section from WT-HF. Dark staining surrounding large lipid containing cells indicate inflammatory cell accumulation in WT-HF. These changes were not present in LCC1-HF. Representative slides are shown.
Overexpression of hepatic CEACAM1 reduced NASH pathogenesis: Mice on HF diet for more than 16 weeks exhibit features of NASH, a more progressive form of steatosis (Abdeen et al., 2006; McClain et al., 2004). Accordingly, H&E staining of liver sections revealed scattered inflammatory cell infiltration in WT mice fed HF for 4 months. (Figure 3.5A, upper right panel, red circle) and altered hepatocellular architecture with microsteatosis and macrosteatosis (Figure 3.5A, upper right panel, arrows). Hepatic CEACAM1 markedly reduced inflammation, as indicated by absence of inflammatory cell islands and normal hepatic architecture in L-CC1 HF (Figure 3.5A, bottom right panel), as in WT RD (Figure 3.5A, upper left panel). Overexpression of hepatic CEACAM1 reduced adipocyte size and inflammation in WAT: Mice on HF diet for more than 16 weeks exhibit WAT inflammation. Accordingly, H&E staining of WAT sections revealed crown-like structures of inflammatory cell infiltration in WT mice fed HF for 4 months. (Figure 3.5B, upper right panel) and altered adipocyte architecture with very large lipid filled adipocytes (Figure 3.5B, upper right panel). Hepatic CEACAM1 markedly reduced inflammation, as indicated by absence of crown-like structures and normal adipocyte architecture and size in L-CC1 HF (Figure 3.5B, bottom right panel), as in WT RD (Figure 3.5B, upper left panel).

Compensatory β-cell proliferation without fasting hyperinsulinemia or blunted insulin secretion in L-CC1 mice. To further understand the protection of LCC1 mice from fasting hyperglycemia and hyperinsulinemia, we examined insulin secretion after 21 weeks of HF. Unlike WT, which develop fasting hyperinsulinemia (Table 3.4, Figure 3.6A, time 0 min., open red squares) and the appearance of blunted first phase insulin secretion (Figure 3.6A, time 2 min., open red squares), LCC1 mice are protected from HF induced
fasting hyperinsulinemia (Figure 3.6A, time 0 min., open green circles compared to solid red squares) and blunted first phase insulin secretion (Figure 3.6A, time 2 min.).

Additionally, quantified sections of pancreata after 31 weeks of HF in WT revealed insignificant compensatory beta cell proliferation (Figure 3.6B, shaded red bar, top right panel), suggesting beta cell failure in association with fasting hyperglycemia. In contrast to WT, LCC1 on HF had a robust compensation to high-fat feeding (Figure 3.6B, shaded green bar, bottom right panel).

**FIG 3.6:** Compensatory β-cell proliferation without fasting hyperinsulinemia or blunted insulin secretion in L-CC1 mice. Mice (n>6 per feeding group per mouse group) were fed RD or HF for the indicated time periods. (A) Insulin secretion: Each mouse was anesthetized prior to receiving IP glucose injection (3 mg/kg body weight) and their blood glucose level was determined at 0-30 minutes post-injection. (B) Beta cell area: Pancreata were fixed, paraffin embedded, sectioned and labeled with insulin antibody (blue) prior to quantification of beta cell percentage of total pancreas area in each field. *P<0.05 HF (open red squares-WT, or green circles-L-CC1) vs RD (closed red squares-WT, or green circles-L-CC1).
PPARα-dependent downregulation of CEACAM1 by HF diet. In agreement with other reports (de Fourmestraux et al., 2004), HF induces hepatic PPARα mRNA after 9 days but does not alter its protein content until after 30 days (~3- to 4-fold) (not shown). Switching to RD restores PPARα mRNA and protein content. Because PPARα activation by fatty acids plays an important role in diet-induced insulin resistance (Kersten et al., 1999; Leone et al., 1999; Patsouris et al., 2006), we examined whether the regulation of Ceacam1 by HF implicates PPARα activation.

Feeding 7 week-old male BL6 mice a diet supplemented with a PPARα agonist, Wy14,643 (Wy) for 3 days increases hepatic PPARα protein levels (Fig. 3.7A) and its activity, as assessed by elevation of the protein (Fig. 3.7A) and mRNA levels (not shown) of its target genes, including CD36/FABP. As expected, PPARα activation lowers hepatic and serum triglyceride levels (Table 3.5). Consistent with reduction in hepatic CEACAM1 content (Fig. 3.7A), PPARα activation decreases insulin clearance (C/I; Table 3.5). Consistent with decreased insulin secretion as a result of enhanced fatty acid oxidation in β-cells upon PPARα activation (Gremlich et al., 2005; Rubi et al., 2002), C-peptide levels were ~5-fold lower in Wy-fed than chow-fed mice (Table 3.5). This suggests that CEACAM1-dependent insulin clearance is reduced by PPARα activation to compensate for the diminished insulin secretion and limit insulin deficiency (as assessed by mildly reduced insulin levels). This maintains normal glucose homeostasis (Table 3.5) and insulin sensitivity, as demonstrated by intact insulin-stimulated IRβ phosphorylation in skeletal muscle and liver (not shown).
FIG. 3.7 High-fat feeding alters hepatic CEACAM1 levels via a PPARα-dependent mechanism. (A) 2 month-old BL6 mice (n>4) were fed a chow diet supplemented with Wy14,643 (Wy) for 3 days and their livers removed for Western analysis of protein content of CEACAM1 (CC1), PPARα and its target CD36. (B) 2 month-old BL6.PPARα−/− and PPARα+/+ mice (n>8) were fed RD or HF for 30 days and subjected to glucose tolerance test (ii) before Western analysis of hepatic CEACAM1 content (i), as in panel A above. *P<0.05 HF (solid circles) vs RD (open squares). (C) CEACAM1 metabolic regulation in a fasting-refeeding paradigm. Mice were fed ad libitum (Random, R) or fasted (F) overnight by withdrawing food at 5:00 p.m the previous day and then refed ad libitum for 1-24h before analyzing their hepatic PPARα mRNA content (i) or FAS and CEACAM1 protein levels (ii). β-actin cDNA and α-tubulin antibody were used for normalization. Gel represents > 3 mice per each time point. Liver glycogen content was also assayed relative to wet tissue weight. n> 5 mice per each time point. Data are presented as ± SEM.
Table 3.5: Effect of Wy14,643 on serum and tissue biochemistry in C57BL/6 male mice. Male mice (2 months of age; n>4 per group) were fed for 3 days a standard chow or a chow mixed at a concentration of 0.1% w/w with Wy14,643 (Wy). *P<0.05 Wy versus Chow. Values are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Chow Diet</th>
<th>Wy-Diet</th>
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<tbody>
<tr>
<td>Hepatic Triglyceride (μg/mg protein)</td>
<td>123. ± 9.0</td>
<td>80. ± 10. *</td>
</tr>
<tr>
<td>Hepatic Cholesterol (μg/mg)</td>
<td>7.2 ± 0.5</td>
<td>7.5 ± 0.3</td>
</tr>
<tr>
<td>Fasting Serum TG (mg/dl)</td>
<td>70. ± 8.2</td>
<td>43. ± 9.3 *</td>
</tr>
<tr>
<td>Fasting Serum Insulin (pM)</td>
<td>464. ± 18.9</td>
<td>354. ± 10.6 *</td>
</tr>
<tr>
<td>Fasting Serum C-peptide (pM)</td>
<td>488. ± 95.3</td>
<td>99.2 ± 11.8 *</td>
</tr>
<tr>
<td>Steady State C-Peptide/Insulin</td>
<td>1.0 ± 0.2</td>
<td>0.3 ± 0.0 *</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mg/dl)</td>
<td>90. ± 11.</td>
<td>82. ± 8.8</td>
</tr>
<tr>
<td>Fasting Serum FFA (mEq/l)</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.0</td>
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In support of the negative effect of PPARα activation on CEACAM1, 30 day-HF fails to reduce CEACAM1 mRNA (not shown) and protein levels in BL6.PPARα−/− mice (Fig. 3.7Bi). Accordingly, HF does not reduce insulin clearance in these mice, but instead increases it, most likely to compensate for the rise in insulin secretion, as suggested by the ~2-fold increase in C-peptide level (not shown). As previously shown (Guerre-Millo et al., 2001), this maintains normal insulin and glucose levels (not shown), in addition to glucose tolerance (Fig. 3.7Bii).

As has been reported (Patsouris et al., 2006), HF elevates hepatic protein content of PPARγ in both PPARα−/− and PPARα+/+ mice (Fig. 3.7Bii). Because hepatic CEACAM1 content in PPARα−/− mice is protected against HF, elevation of hepatic PPARγ content suggests that activation of PPARα, but not PPARγ, downregulates hepatic CEACAM1. These findings are consistent with the more dominant expression of CEACAM1 and
PPARα in the hepatocyte, as opposed to the adipocyte, a site with a negligible expression of CEACAM1 (Accili et al., 1986).

CEACAM1 is regulated by fasting/refeeding. Western analysis shows that hepatic CEACAM1 level is low at fasting (F), when the level of PPARα mRNA is highest (Fig. 3.7Ci & ii) and its activation by the high supply of fatty acids during lipolysis is robust. By contrast, CEACAM1 levels peak at 4 and 7-8 hours of refeeding when insulin surges (Najjar et al., 2005b). This is consistent with increase in Ceacam1 promoter activity by insulin (Najjar et al., 1996).

In agreement with the reported changes in mRNA levels (Assifi et al., 2005; Iritani, 1992), FAS protein content progressively increases during refeeding at a high CEACAM1:FAS ratio until about 8 hours, at which point, the stoichiometry is reversed (Fig. 3.7Cii). Given that CEACAM1 mediates a negative effect of insulin on FAS activity at 4 and 7 hours of refeeding (Najjar et al., 2005b), it is possible that the higher CEACAM1:FAS ratio maintains low FAS activity in the first few hours of refeeding (Assifi et al., 2005; Iritani, 1992). This contributes to the regulation of the FAS substrate, malonyl-CoA, and hence, fatty acid transport to mitochondria for β-oxidation, an essential step in glycogen repletion. In agreement with other reports (Roden and Shulman, 1999), this takes ~8 hours of refeeding (Fig. 3.7C bar graph).

PPARα activation represses Ceacam1. Similar to its in vivo effect, Wy14,643 (Wy) decreases Ceacam1 mRNA and protein content in rat hepatoma FAO cells beginning at 1 and 2 hours, respectively, (Fig. 3.8A). This rapid negative regulatory effect suggests that Ceacam1 is transcriptionally regulated by PPARα and that the effect of Wy on Ceacam1 expression in murine liver is due to PPARα rather than confounding metabolic factors.
FIG. 3.8: Molecular analysis of PPARα repression of Ceacam1 expression. (A) Rat hepatoma FAO cells were treated with Wy,14,463 (Wy) for 0-24 hours (h) prior to Northern and Western analyses of Ceacam1 levels. (B) A series of rat Ceacam1 promoter fragments with different 5' ends (nt –1609, –439, –249) and a common 3' end (nt –21) was ligated into the pGL3 promoterless plasmid, transiently co-transfected with renilla luciferase (pRL) into H4-II-E rat hepatoma cells (i) and HEK293 cells (ii), treated with Wy (100µM in hepatoma cells and 0-500µM in HEK cells), and luciferase activity assayed and determined relative to that in Renilla. For comparison, luciferase activity in cells transfected with the reverse promoter was included. Transfections were performed in triplicates and mean ± s.d in relative light units of each construct is graphically shown. The relative location of the 2 potential PPARα response elements (PPRE) in the –1609 promoter fragment are schematically shown above. (C) PPARα+/+ and PPARα−/− mice were fed a Wy-supplemented diet prior to liver extraction and ChIP analysis, as described in Methods (i). The relative location of the fragment amplified using the proximal (–548/–282) and distal (+6499/+6764) primers in the Ceacam1 (Cc1) gene are also shown. Malic enzyme, a positive target of PPARα, was used as control. The gel represents experiments on >3 mice per treatment category per mouse group. ChIP analysis was performed on livers derived from HF-fed BL6 mice (n>2 per feeding group), but quantitated by qPCR and normalized relative to input (ii). The graph includes representatives of the lowest and highest fold enrichment of PPARα binding to the Ceacam1 promoter in both feeding groups.
Using a luciferase reporter assay, we show that: 1) Wy treatment reduces the promoter activity by ~4-fold in rat hepatoma H4-II-E cells (Fig. 3.8Bi, Wy- vs vehicle (Veh)-treated –1609pLuc), and 2) deleting the genomic DNA region containing both PPARα response elements (PPRE) between nts –439 and –249 (Najjar et al., 1996) reverses the effect of PPARα activation on rat Ceacam1 promoter activity (Fig. 3.8Bi, Wy-treated –249pLuc vs Veh-treated –1609pLuc). Similarly, Wy treatment causes a reducing effect on the promoter activity of the rat Ceacam1 promoter (~1609pLuc) in human embryonic kidney (HEK293) cells (Fig. 3.8Bii), with an expected lower potency than in rat cells (Cariello et al., 2005; Evans et al., 2004).

Chromatin immunoprecipitation (ChIP) assay using a proximal primer (PP) set spanning a mouse Ceacam1 promoter between nts –280 and –562 shows that liganded PPARα binds to Ceacam1 gene in liver lysates derived from Wy- but not Veh-fed PPARα+/+ mice or Wy-fed PPARα−/− mice (Fig. 3.8Ci). Similar observation is made for the positive control, malic enzyme, a known target of PPARα. The distal primer (DP) set amplifying a region between nts +6499 and +6764 in Ceacam1 gene does not detect any binding. As opposed to RD-fed mice, HF intake for 30 days amplifies binding of PPARα to the Ceacam1 gene by ~4 to 14-fold relative to input (Fig. 3.8Cii). The data reveal that activated PPARα binds to Ceacam1 gene to downregulate its transcription in rodents and humans.
3.5 Discussion

The current studies show that hyperinsulinemia resulting from altered CEACAM1-dependent insulin clearance causes insulin resistance and visceral obesity in response to short-term fat intake before a robust pro-inflammatory state develops.

High-fat intake for 30 days reduces hepatic CEACAM1 levels by >50% in BL6 (and BL6xFVB) mice. Consistent with Cc1\textsuperscript{+/−} phenotype (DeAngelis et al., 2008b), this loss-of-function of Ceacam1 impairs insulin clearance and leads to hyperinsulinemia and hepatic insulin resistance. Conversely, gain-of-function of hepatic Ceacam1 protects against hyperinsulinemia, insulin resistance and visceral obesity in response to a 30 day-fat intake. These observations suggest that hyperinsulinemia caused by impaired insulin clearance, plays a causative role in the early pathogenesis of diet-induced obesity and insulin resistance.

Our finding of a progressive increase in fat mass beginning at ~ 9 days, followed by hyperinsulinemia and insulin resistance (30 days) is in agreement with other recent reports on dietary obesity in BL6 mice (Kim et al., 2008; Park et al., 2005). The Schwartz laboratory (Kim et al., 2008) showed that 60% fat diet induces hyperinsulinemia on BL6 mice before the development of cellular insulin resistance in liver and skeletal muscle (4-8 weeks), and especially in WAT, which takes ~14 weeks to develop in correlation with systemic and WAT-associated inflammatory state. While this differs slightly from our findings and those of the Kim laboratory (Park et al., 2005) of a concurrent development of hyperinsulinemia with whole body and hepatic insulin resistance, it provides proof-of-principle that hyperinsulinemia precedes altered insulin signaling in peripheral insulin
target tissues, including liver, and supports our finding of intact insulin signaling in WAT after 30 days of fat intake. Given that inducible expression of CEACAM1 protects against insulin resistance, hyperinsulinemia and visceral obesity, we propose that high-fat diet initially reduces hepatic CEACAM1 and subsequently, impairs insulin clearance to cause hepatic insulin resistance and lipogenesis. This leads to lipid redistribution to WAT to give rise to visceral adiposity, and to the skeletal muscle where it reduces glucose uptake and induces lipotoxicity (Muoio and Koves, 2007).

The model above emphasizes that dietary fat initiates a cascade of metabolic abnormalities commencing with hyperinsulinemia, which results from a PPARα-dependent reduction of CEACAM1 and its function in hepatic insulin clearance. Because CEACAM1 decreases FAS activity (Najjar et al., 2005b), it is possible that downregulation of CEACAM1 by dietary fat constitutes a positive feedback mechanism on fatty acid β-oxidation, not only by supplying newly synthesized fatty acids to activate PPARα (Chakravarthy et al., 2005), but also by removing the inhibitory effect of malonyl-CoA on fatty acid transport into the mitochondria. This hypothesis is supported by the marked decrease of CEACAM1 level at fasting, an event that involves an accelerated shift from glycolytic to lipolytic metabolism and a robust PPARα activation (Durgan et al., 2006; Patsouris et al., 2006). In the early few hours of refeeding (~8 hours), PPARα plays a significant role in maintaining β-oxidation during the stepwise reversal of the fasting metabolic state by insulin surges until glycogen stores are replenished (Sugden et al., 2002), at which point, malonyl CoA levels are restored, β-oxidation stops and glycolysis resumes (Zammit and Moir, 1993). Thus, it appears that the coordinated regulation by PPARα and insulin positions CEACAM1 at a favorable
stoichiometry relative to FAS to inhibit its activity and take part in the metabolic adaptation during the fasting-to-refeeding transition.

Downregulation of Ceacam1 expression by PPARα is demonstrated by: 1) Reduction in the promoter activity of rat Ceacam1 by Wy treatment of rat hepatoma cells and human embryonic kidney cells; 2) rapid decline of Ceacam1 mRNA and protein content upon treating hepatoma cells or feeding mice with Wy; 3) binding of liganded PPARα to Ceacam1 promoter in liver lysates derived from wild-type, but not PPARα−/− mice in which CEACAM1 resist a decrease by HF intake, and 4) induction of PPARα binding to Ceacam1 promoter in liver lysates derived from HF-fed mice. Our observations are consistent with recent reports on the downregulation of Ceacam1 mRNA in mice treated with novel more highly PPARα-selective piperidine agonists that are more potent activators of PPARα and exert a stronger lipid-lowering effect than fibrates, which are weak and non-selective PPARα agonists (Kane et al., 2009). Although PPARα upregulates genes involved in fatty acid catabolism, it downregulates the expression of many liver-specific genes involved in glucose metabolism, cell adhesion, CYP2C family of steroid hydroxylases and positive acute-phase response genes induced during inflammation (Kane et al., 2009; Shah et al., 2007). Further studies are needed to delineate the mechanisms involved in Ceacam1 repression in rodents and human cells, but ChIP analysis suggests that liganded PPARα directly regulates Ceacam1 expression.

The data provide an in vivo demonstration that PPARα-dependent reduction of hepatic CEACAM1 levels by fat intake causes hyperinsulinemia, which in turn, triggers various metabolic derangements and promote visceral obesity without initiating a systemic inflammatory response. This provides proof-of-principle that hyperinsulinemia
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Chapter 4

Discussion

We have previously shown that rats with low aerobic running capacity (low capacity runners - LCR) exhibit the metabolic syndrome, including hyperinsulinemia, hypertriglyceridemia, visceral obesity, and body weight gain. Moreover, hyperinsulinemia in LCR stems from compromised insulin clearance, which is in turn, associated with reduction in hepatic protein, carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-1 mRNA levels as compared to rats with high aerobic running capacity (high capacity runners - HCR) (Wisloff et al., 2005).

Whether insulin resistance precedes or follows obesity in type 2 diabetes is unresolved (Srinivasan et al., 2003). However, LCR develop hyperinsulinemia and hypertriglyceridemia at 5 weeks of age, before visceral obesity and body weight increase (Wisloff et al., 2005), demonstrating a key role for altered insulin and triglyceride homeostasis in the abnormal metabolic state in this model. The marked reduction of hepatic CEACAM1 in LCR and the similarity in the phenotype to that in Ceacam1 mutants (L-SACC1 and Cc1<sup>−/−</sup> mice) (DeAngelis et al., 2008a; Park et al., 2006; Poy et al., 2002b) provide circumstantial evidence that altered CEACAM1-dependent insulin clearance pathways link the pathogenesis of hyperinsulinemia to liver steatosis, hypertriglyceridemia and visceral obesity in LCR.
The role of CEACAM1 in this process is further supported by the observation that caloric restriction, which induces hepatic CEACAM1 expression, is more effective in regulating insulin and lipid metabolism in this model than endurance training, which exerts a milder effect on hepatic CEACAM1 expression. In this regard, endurance training does not fully reduce hepatic and serum triglyceride levels, in parallel to persistently elevated hepatic FAS protein content, which most likely results from chronically elevated levels of insulin in trained LCR. That training does not restore insulin and lipid metabolism is in agreement with other reports showing that it fails to reduce hyperinsulinemia and hepatic FAS mRNA levels in obese Zucker rats, in spite of decreasing body weight and total fat mass (Fiebig et al., 2002). The reversal effect of exercise on lipid metabolism and insulin action in skeletal muscle and WAT of LCR (Haram et al., 2009) precludes the possibility that any positive effect of this program on liver was diminished in the 48 hours after the last bout of exercise before sacrifice. This provides evidence that endurance program indeed exerts a preferential role on metabolism in skeletal muscle, as opposed to caloric restriction, which primarily affects insulin action and metabolism in liver.

Our observations of a significant lowering effect of caloric restriction in comparison to exercise on hepatic steatosis are supported by human studies indicating that caloric restriction alone, without exercise, reduces liver triglyceride in a small cohort of obese individuals (Larson-Meyer et al., 2008). While these studies did not subject a group of obese humans to an exercise program alone, they serve to emphasize the beneficial therapeutic role of caloric restriction on lipid metabolism in liver.
The mechanism underlying the regulatory effect of caloric restriction on CEACAM1 expression is not well delineated. However, we have shown an inverse effect of high-fat intake and mobilization of fatty acids from WAT during fasting on CEACAM1 transcription (Chapter 3). The negative effect of CEACAM1 on FAS enzymatic activity (Najjar et al., 2005a) implicates it in the regulation of the level of malonyl-CoA, a key determinant of fatty acid transport to mitochondria for β-oxidation. Thus, it is likely that under conditions of caloric restriction, CEACAM1 is elevated to reduce insulin levels in LCR, and consequently, hepatic FAS content. Together with inhibiting FAS enzymatic activity, this elevates malonyl-CoA levels to reduce β-oxidation, and ultimately, gluconeogenesis. This notion is supported by inactivation of the peroxisome proliferator-activated receptor α (PPARα), as suggested by reduced hepatic levels of one of its targets, CD36, and the reduction of fasting blood levels by caloric restriction.

As recently reported (Thyfault et al., 2009), LCR exhibit features of NASH, including steatohepatitis, fibrosis and elevation in oxidative stress. In agreement with other reports showing improvement of hepatic histology and reversal of hepatic steatosis and insulin resistance with a >9% loss of body weight by dietary restriction in humans (Harrison et al., 2009), caloric restriction reverses most, if not all NASH-characteristic features of LCR. Given the cumulative effect of increased fat deposition in liver and macrophage infiltration from WAT on the hepatic inflammatory milieu (Bigorgne et al., 2008; Sheth et al., 1997), it is not all that surprising that LCR develop steatohepatitis. CEACAM1 also acts as a co-inhibitory receptor after T cell activation to inhibit inflammation (Gray-Owen and Blumberg, 2006). Thus, it is likely that reduction in
hepatic CEACAM1 levels predisposes LCR to develop a more progressive form of hepatic steatosis. Consistent with the partial dependence of the anti-inflammatory function on the phosphorylation of immunoreceptor tyrosine-based inhibition motifs within the cytoplasmic domain of CEACAM1 (Nagaishi et al., 2006), L-SACC1 mice exhibit key features of NASH when exposed to conditions that trigger inflammation, such as high-fat feeding (Lee et al., 2008). The development of NASH in LCR as in L-SACC1 mice, and conversely, the complete reversal of the NASH-characteristic phenotype by caloric restriction in parallel to inducing hepatic CEACAM1 levels lend further support to the role of CEACAM1-dependent pathways in the pathogenesis of the altered metabolic state in LCR.

In summary, the current studies demonstrate that although exercise alone improves insulin action in muscle and WAT, and reduces body weight in LCR (Haram et al., 2009), caloric restriction primarily targets insulin and lipid metabolism in liver. Although a cause-effect relationship is not well delineated, our results point to a differential inducing effect on hepatic CEACAM1 levels by caloric restriction as compared to exercise. Further studies are required to identify the CEACAM1-dependent pathways that underlie the beneficial effect of caloric restriction on hepatic metabolism. However, using this unique animal model of metabolic syndrome, this study provides suggestive evidence that CEACAM1 constitutes an important pharmacological target to treat this disease.

High-fat intake for 30 days reduces hepatic CEACAM1 levels by >50% in BL6 (and BL6xFVB) mice. Consistent with Cc1−/− phenotype (DeAngelis et al., 2008b), this loss-of-function of Ceacam1 impairs insulin clearance and leads to hyperinsulinemia and
hepatic insulin resistance. Conversely, gain-of-function of hepatic Ceacam1 protects against hyperinsulinemia, insulin resistance and visceral obesity in response to a 30 day-fat intake. These observations suggest that hyperinsulinemia caused by impaired insulin clearance, plays a causative role in the early pathogenesis of diet-induced obesity and insulin resistance.

Our finding of a progressive increase in fat mass beginning at ~ 9 days, followed by hyperinsulinemia and insulin resistance (30 days) is in agreement with other recent reports on dietary obesity in BL6 mice (Kim et al., 2008b; Park et al., 2005b). The Schwartz laboratory (Kim et al., 2008b) showed that 60% fat diet induces hyperinsulinemia on BL6 mice before the development of cellular insulin resistance in liver and skeletal muscle (4-8 weeks), and especially in WAT, which takes ~14 weeks to develop in correlation with systemic and WAT-associated inflammatory state. While this differs slightly from our findings and those of the Kim laboratory (Park et al., 2005b) of a concurrent development of hyperinsulinemia with whole body and hepatic insulin resistance, it provides proof-of-principle that hyperinsulinemia precedes altered insulin signaling in peripheral insulin target tissues, including liver, and supports our finding of intact insulin signaling in WAT after 30 days of fat intake. Given that inducible expression of CEACAM1 protects against insulin resistance, hyperinsulinemia and visceral obesity, we propose that high-fat diet initially reduces hepatic CEACAM1 and subsequently, impairs insulin clearance to cause hepatic insulin resistance and lipogenesis. This leads to lipid redistribution to WAT to give rise to visceral adiposity, and to the skeletal muscle where it reduces glucose uptake and induces lipotoxicity (Muoio and Koves, 2007).
The model above emphasizes that dietary fat initiates a cascade of metabolic abnormalities commencing with hyperinsulinemia, which results from a PPARα-dependent reduction of CEACAM1 and its function in hepatic insulin clearance. Because CEACAM1 decreases FAS activity (Najjar et al., 2005b), it is possible that downregulation of CEACAM1 by dietary fat constitutes a positive feedback mechanism on fatty acid β-oxidation, not only by supplying newly synthesized fatty acids to activate PPARα (Chakravarthy et al., 2005a), but also by removing the inhibitory effect of malonyl-CoA on fatty acid transport into the mitochondria. This hypothesis is supported by the marked decrease of CEACAM1 level at fasting, an event that involves an accelerated shift from glycolytic to lipolytic metabolism and a robust PPARα activation (Durgan et al., 2006; Patsouris et al., 2006). In the early few hours of refeeding (~8 hours), PPARα plays a significant role in maintaining β-oxidation during the stepwise reversal of the fasting metabolic state by insulin surges until glycogen stores are replenished (Sugden et al., 2002), at which point, malonyl-CoA levels are restored, β-oxidation stops and glycolysis resumes (Zammit and Moir, 1993). Thus, it appears that the coordinated regulation by PPARα and insulin positions CEACAM1 at a favorable stoichiometry relative to FAS to inhibit its activity and take part in the metabolic adaptation during the fasting-to-refeeding transition.

Downregulation of Ceacam1 expression by PPARα is demonstrated by: 1) Reduction in the promoter activity of rat Ceacam1 by Wy treatment of rat hepatoma cells and human embryonic kidney cells; 2) rapid decline of Ceacam1 mRNA and protein content upon treating hepatoma cells or feeding mice with Wy; 3) binding of liganded PPARα to Ceacam1 promoter in liver lysates derived from wild-type, but not PPARα−/− mice in
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Chapter 5

Summary

The first study (Chapter 2) shows that caloric restriction (CR) fully reverses the altered hepatic metabolic state in ad libitum (AL) fed LCR, and that this implicates the recovery of hepatic CEACAM1 and its ensuing regulatory effect on insulin clearance. In contrast, endurance training fails to fully restore hepatic CEACAM1 content, and serum insulin and triglyceride levels.

These are the primary results:

1) Caloric restriction reduced body mass and visceral obesity in LCR. This was accomplished by subjecting LCR to 30% caloric restriction for 3 months. Body weight was reduced by 20% and visceral fat by 50%.

2) Caloric restriction improved hepatic insulin clearance and overall insulin sensitivity in LCR. This was demonstrated by decreased (to HCR levels) hyperinsulinemia, and increasing c-peptide/insulin ratio and increasing CEACAM1 protein levels. We also demonstrated improvements in glucose and insulin tolerance.

3) Caloric restriction improved lipid metabolism in LCR. This was demonstrated by decreased fatty acid synthase protein, CD36 protein, hepatic and serum triglyceride, and serum free fatty acid. All these levels were reduced to the level of HCR.
4) Caloric restriction reduced NASH pathogenesis in LCR. This was demonstrated by hemotoxylin and eosin stained liver sections, showing micro-and macro-lipid droplets, and inflammatory cell infiltrates, and by sirius red staining showing chicken-wire pattern of fibrosis in AL-LCR, but not in CR-LCR or AL-HCR. We also demonstrated reduction in hepatic lipid peroxidation by Thiobarbituric acid reactive substances (TBARS) assay of CR-LCR to AL-HCR level.

5) There was a lack of metabolic effect of endurance training in LCR liver. This was demonstrated by non-reduced hepatic and serum triglyceride, fatty acid synthase protein, serum insulin, and lipid peroxidation (by TBARS), and non-increased c-peptide/insulin ratio, CEACAM1 protein and CEACAM1 phosphorylation levels in trained compared to sedentary LCR, with sedentary HCR as control.

The second study (Chapter 3) shows that hyperinsulinemia resulting from altered CEACAM1-dependent insulin clearance causes insulin resistance and visceral obesity in response to short-term fat intake before a robust pro-inflammatory state develops.

These are the primary results:

1) High fat diet (HF) reduces hepatic CEACAM1 levels and impairs insulin clearance. An increase in visceral adiposity was noted in wild-type mice after 9 days of high fat-feeding. CEACAM1 mRNA and protein levels were reduced by more than 50% after 30, but not 9 days of HF. C-peptide/insulin molar ratio was decreased after 30, but not 9 days of HF.

2) HF causes hepatic insulin resistance in a CEACAM1-dependent manner. After HF for 24 to 30 days, but not 12 to 15 days, wild-type mice have glucose intolerance and
delayed glucose recovery following insulin injection during insulin tolerance test, and decreased phosphorylation of the insulin receptor β-subunit by insulin in liver lysates.

3) HF causes chronic hyperinsulinemia resulting in increased hepatic lipogenesis. After 30 days HF, wild-type mice had fasting hyperinsulinemia, increased hepatic fatty acid synthase and CD36 protein levels, increased hepatic triglyceride, increased white adipose tissue (WAT) fatty acid transporter-1, normal IRβ and Akt phosphorylation in response to insulin in WAT, normal IRβ but decreased Akt phosphorylation in response to insulin in soleus muscle.

4) HF for 30 days does not cause a robust inflammatory response. After 30 days HF, wild-type mice had unchanged macrophage markers and adipokines.

5) Liver-specific overexpression of CEACAM1 protects against diet-induced insulin resistance. Mice were generated with liver-specific overexpression of CEACAM1 (termed L-CC1). After 30 days of HF, wild-type mice had increased visceral fat, hyperinsulinemia, hypertriglyceridemia, decreased c-peptide/insulin ratio, decreased CEACAM1 protein and mRNA, decreased IRβ phosphorylation in response to insulin in liver, and glucose and insulin intolerance. In contrast, L-CC1 mice maintained 5 to 6 times normal level of CEACAM1, and were protected from increased visceral fat, hyperinsulinemia, hypertriglyceridemia, decreased c-peptide/insulin ratio, decreased IRβ phosphorylation in response to insulin in liver, and glucose and insulin intolerance.

6) Liver-specific overexpression of CEACAM1 protects against long-term high fat diet-induced metabolic abnormalities. After 4 to 6 months of HF, wild-type mice had increased visceral fat, large adipocytes, hyperinsulinemia, hypertriglyceridemia,
decreased c-peptide/insulin ratio, decreased CEACAM1 protein and mRNA, glucose and insulin intolerance, and hepatic micro-and macro lipid droplets with inflammatory cell infiltration. In contrast, L-CC1 mice maintained 5 to 6 times normal level of CEACAM1, and were protected from increased visceral fat, large adipocytes, hyperinsulinemia, hypertriglyceridemia, decreased c-peptide/insulin ratio, glucose and insulin intolerance, and hepatic micro-and macro lipid droplets with inflammatory cell infiltration.

7) Liver-specific overexpression of CEACAM1 produces compensatory β-cell proliferation without fasting hyperinsulinemia or blunted insulin secretion in L-CC1 mice. After 4 to 6 months of HF, wild-type mice had blunted first phase insulin secretion and insignificant compensatory beta-cell proliferation. In contrast, L-CC1 compensated to high-fat feeding with preserved first phase insulin secretion and compensatory beta-cell proliferation.

8) High fat diet produces PPARα-dependent downregulation of CEACAM1. After 30 days of HF, wild-type mice have increased hepatic PPARα mRNA and protein. Feeding 7 week-old male BL6 mice a diet supplemented with a PPARα agonist, Wy14,643 (Wy) for 3 days increases hepatic PPARα protein levels, and its target genes, including CD36/FABP, decreases hepatic and serum triglyceride levels, decreases c-peptide/insulin ratio, produces normal insulin-stimulated IR phosphorylation in skeletal muscle. After 30 days of HF, BL6.PPARα mice had normal CEACAM1 levels, increased c-peptide, increased c-peptide/insulin ratio, normal glucose tolerance, elevated hepatic protein content of PPARα in both PPARα and PPARα mice. The level of PPARα during fasting is high and during feeding is low; whereas the level of CEACAM1 peaks at 4 and 7-8 hours during insulin surges.
9) PPARα activation represses Ceacam1. Wy decreases CEACAM1 in FAO cells at 1 to 2 hours. Luciferase reporter assay shows Wy reduces Ceacam1 promoter activity in rat hepatoma H4-II-E cells only when PPARα response elements (PPRE) are present. Wy reduces rat Ceacam1 promoter activity in human embryonic kidney cells. Chromatin immunoprecipitation assay shows liganded PPARα binds to Ceacam1 gene in liver lysates from Wy fed wild-type mice but not PPARα−/− mice. HF intake for 30 days amplifies binding of PPARα to the Ceacam1 gene.
Chapter 6

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