Reduction of Hepatic CEACAM1 Levels: An Early Mechanism of Diet-induced Insulin Resistance

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Reduction of Hepatic CEACAM1 Levels:
an Early Mechanism of Insulin Resistance Induced by
High-Fat Diet

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DEDICATION

I dedicate this work to my major advisor, Dr. Sonia M. Najjar for her continuous guidance and help, to my parents and my siblings for their great love, support and prayers.

I also dedicate this dissertation to my sweet, loving and supportive wife, Rasha Gharibeh, for her continuous encouragement during the writing of this thesis.

Last, but not least, I dedicate this thesis to our son, Saif, who has become a principle driving force in my life.
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INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) affects about 20.8 million people in the United States (American Diabetes Association, 2006) and is often linked to obesity which is considered an insulin-resistance state (Kopelman, 2000). Almost 20% of adults in the United States are classified as obese and 7.3% as having diabetes in 2000 (Camp et al., 2002). The prevalence of the two diseases has grown over the past decade, with a 61% increase in obesity and a 49% increase in T2DM (Camp et al., 2002). Both diseases are of great importance because of the serious complications they cause, and the increasingly high number of patients affected by both diseases.

There is a strong correlation between insulin resistance in T2DM, high fat (HF) intake and obesity (Kopelman, 2000). High plasma free fatty acids (FFA) that are released from the white adipose tissue (WAT) of obese people affect insulin action, and play an important role in the pathogenesis of insulin resistance and T2DM. Adipose tissue is now envisioned as an endocrine tissue (Heilbronn et al., 2004) that not only releases FFA, but also secretes hormones and factors, collectively known as adipokines, that affect insulin action such as leptin, adiponectin, TNF-α, IL-1β, IL-6, resistin and others (Lehrke and Lazar, 2004; Pickup and Crook, 1998; Wellen and Hotamisligil, 2003). Altered fat metabolism and hyperinsulinemia also have a role in the development of other diseases of the cardiovascular system (hypertension, dyslipidemia, cerebrovascular problems, atherosclerosis, and others), the central nervous system (CNS), renal system, skin, and others. It is important to understand the molecular link between
altered fat metabolism in obesity and T2DM, and insulin action. Investigating the mechanisms of insulin resistance is of great importance because it is considered a major risk factor for the development of T2DM (Mantzoros and Flier, 1995) and other complications.

Insulin is a polypeptide hormone that is secreted from the β-cells of the pancreas to decrease glucose production in liver and promote fuel storage as glycogen in liver and muscle, and as triglycerides in adipose tissue. Insulin action starts after it binds to the insulin receptor’s extracellular α-subunit (Accili et al., 2001). This will activate the tyrosine kinase and induce autophosphorylation of the receptor on intracellular tyrosine residues, as well as the phosphorylation of further substrates as members of the insulin receptor (IR) substrates family (IRS-1, -2, -3, -4), Shc, and others (Saltiel and Kahn, 2001). Phosphorylation and activation of these signaling molecules is an essential part in the mechanism of insulin action and to elicit the different functions of the hormone (Le Roith and Zick, 2001; Saltiel and Kahn, 2001; White, 1998). Two main pathways are implicated in insulin action, the PI3-kinase and the Ras/Raf/MAP kinase pathway (White, 1997; White and Kahn, 1994). In the former pathway, insulin regulates several functions including: glucose transport, glycogen synthesis, protein synthesis, anti-lipolysis, anti-apoptosis, and affects gene expression and mitogenesis. Through the latter pathway, insulin works mainly on gene expression and mitogenesis (Le Roith and Zick, 2001).

In T2DM, insulin action is impaired as insulin target tissues become insulin resistant (Accili et al., 2001; Kahn et al., 2000). Insulin resistance is encountered in obese
subjects and is usually associated with increased HF intake. This leads to increase in plasma FFA as well as deranged fat metabolism. This alteration in lipid metabolism contributes to the pathogenesis of insulin resistance and development of T2DM. As a result, insulin action will be impaired in the insulin sensitive tissues; liver, muscle adipose, which reduces their ability to utilize glucose in response to insulin, as well as fatty acid storage in adipose tissue. Indeed, many investigators consider the elevation in plasma FFA levels the primary event in the pathogenesis of insulin resistance.

In addition to FFA release, visceral adipose tissue and recruited macrophages secrete pro-inflammatory mediators that attenuate insulin action and cause insulin resistance. To overcome this resistance, pancreas secretes more insulin, and a state of high absolute levels of insulin, termed hyperinsulinemia, develops (Duckworth et al., 1998). Impaired insulin clearance in liver can cause hyperinsulinemia (Poy et al., 2002b). Hyperinsulinemia as a result of impaired insulin clearance also contributes to insulin resistance. As we have shown in our laboratory, the liver-specific inactivation of hepatic the CarcinoEmbryonic Antigen-related Cell Adhesion Molecule 1 (CEACAM1), a protein that promotes insulin removal in liver, or the loss of this protein impairs insulin clearance and causes chronic hyperinsulinemia and insulin resistance (Poy et al., 2002b).

To further understand the role of CEACAM1 in the development of insulin resistance, we induced visceral obesity by feeding wild-type (WT) mice a HF diet for 9-30 d. As a result, we confirmed that HF-intake for 30 d in mice causes a 50-60% reduction in hepatic CEACAM1 along with altered fat metabolism, impairment of insulin action...
clearance, hyperinsulinemia, and insulin resistance. Interestingly, after 30 d of HF feeding, we did not notice elevation in the pro-inflammatory mediators (such as IL-6, IL-1β) and the macrophage markers (CD68, and F4/80) which are usually recruited after long-term HF-intake and cause insulin resistance. This led us to the conclusion that insulin resistance did not develop due to increased adipokine levels, rather another mechanism that is implicated at an earlier stage of HF intake.

In light of this finding, we focused on what is coming out of visceral adipose tissue other than adipokines and is implicated in the development of insulin resistance. A large body of studies investigated the role of adipose tissue-released FFA in insulin resistance. This is in support of our finding that 30 d of HF raised plasma FFA levels and caused insulin resistance with a concomitant decrease in hepatic CEACAM1 content. We concluded that elevation of plasma FFA, reduction in hepatic CEACAM1, and insulin resistance are tightly related. To investigate this correlation, we inhibited lipolysis from visceral adipose tissue by nicotinic acid in mice in order to prevent release of FFA from WAT and their elevation in the circulation. As a result, we found that inhibiting FFA release in obese mice prevented the loss of hepatic CEACAM1 and prevented the development of insulin resistance.

In this study we identified a new mechanism for the early development of diet-induced insulin resistance through reduction of hepatic CEACAM1 levels by HF diet for 30 d. The elevation of plasma FFA increases the flux into the liver to be oxidized through the peroxisome proliferator-activated receptor α (PPARα), a nuclear factor that increases the transcription of genes encoding proteins involved in fatty acid oxidation and transport.
into liver and its mitochondria). We also found that reduction in hepatic CEACAM1 by HF diet is caused by FFA activation of PPARα, which in turn decreases hepatic CEACAM at the mRNA and protein levels. Consequently, this leads to further altered fat metabolism, hyperinsulinemia, impairment of insulin clearance, and insulin resistance.

We have confirmed the effect of PPARα activation on hepatic CEACAM1 levels both *in vivo* and *in vitro* studies. We tested PPARα activation using several ways: HF feeding in mice, by direct activation of PPARα in mice and rat hepatoma cells (FAO) via the selective PPARα agonist Wy14,643 (Wy), and by fasting and refeeding mice. In all these cases we have observed increased hepatic PPARα levels in parallel with a decrease in CEACAM1 content. In addition, and in order to further support the role of PPARα in CEACAM1 reduction, we fed PPARα null mice (PPARα−/−) a HF diet for 30 d and observed no decrease in CEACAM1 and normal insulin action. This indicates that PPARα regulates CEACAM1 in liver and the deletion of which protects against hyperinsulinemia and developing insulin resistance as CEACAM1 is protected when PPARα is deleted.

We are herein providing a new mechanism for the early development of diet-induced insulin resistance through reduction of hepatic CEACAM1 via PPARα activation by visceral adipose tissue-derived FFA.
Diabetes Mellitus

Diabetes mellitus is the most common endocrine disease that includes a group of common metabolic abnormalities with a common phenotype of hyperglycemia. Its development is associated with a complex interaction of environmental factors, genetic factors and life-style. It develops as a result of insulin deficiency or diminished insulin effectiveness. This insulin deficiency, which could be absolute or relative, affects carbohydrate, protein and fat metabolism as well as water and electrolyte levels. The pathogenesis of T2DM has implicated several molecular mechanisms: insulin receptor signaling, β-cell development, mitochondrial metabolism, carbohydrate production and utilization, fatty acid oxidation, cytokine signaling, adipogenesis, adrenergic signaling and others (Mootha et al., 2003).

The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2003) has suggested that the subject is diabetic if having a fasting plasma glucose level of 126 mg/dl (7.0 mmol/L) or higher; or a random glucose of 200 mg/dl (11.1 mmol/L) or more taken at any time of the day provided that symptoms of diabetes are present or 200 mg/dl (11.1 mmol/L) 2 h after oral glucose tolerance test (The Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003).

Type 2 diabetes mellitus affects about 5% of the Western population, and rates are steadily increasing with the modern life-style (Mokdad et al., 2001). In the US alone it is estimated that 20.8 million children and adults have diabetes, which is approximately 7%
of the total population (American Diabetes Association, 2006). Out of the 20.8 million, approximately 14.6 million are diagnosed as diabetics, 6.2 million remain undiagnosed and about 54 million people are pre-diabetics. The ADA estimated that about 1.5 million new cases of diabetes were diagnosed in people 20 yr and older in 2005. Genetic predisposition has been shown to play an important role in the development of T2DM (Froguel et al., 1993; Guillausseau et al., 1997; Medici et al., 1999; Newman et al., 1987), with varying penetrance and a polygenic nature (Froguel et al., 1993).

Based on the underlying cause of diabetes, it is classified into two types: type 1 and type 2. Type 1 diabetes mellitus (T1DM), previously know as childhood-onset diabetes, juvenile onset or insulin-dependent diabetes mellitus (IDDM), results from autoimmune destruction of pancreatic β-cells that usually leads to absolute insulin deficiency. It can be immune-mediated or idiopathic. The T1DM patients (about 5-10% of total diabetic population) must be provided with exogenous insulin to be able to carry out the related metabolic processes and prevent ketoacidosis. Although the onset of T1DM occurs mostly in childhood or in early adolescence, it can develop at any age later in life. Patients usually are non-obese and there is no strong evidence that it is genetically linked.

The most prevalent type of diabetes is type 2 diabetes mellitus (T2DM), also known as “adult onset diabetes.” It is a heterogeneous group of disorders that exhibit relative insulin deficiency, and is usually associated with obesity, insulin resistance, impaired insulin secretion, and increased hepatic glucose production (HGP). It usually starts 4 to 7 yr before a definite diagnosis (Harris et al., 1992) and is caused by distinct
genetic and metabolic defects in insulin action and/or secretion. It was previously known as noninsulin-dependent diabetes mellitus (NIDDM) because it can be controlled with oral antidiabetic drugs; however, later on when the disease progresses it becomes inevitable to use insulin to control glycemia in many diabetic patients.

Type 2 diabetes is one of the main health problems in the 21st Century due to many factors, including aging of the population, increased consumption of unhealthy food, increased obesity and the sedentary life style due to lack of exercise. The World Health Organization (WHO) announced that diabetes is an epidemic. In 1985, there were about 30 million people around the world with diabetes (Smyth and Heron, 2006). This number jumped to 135 million in 1995, and to 217 million by the year 2005 (Smyth and Heron, 2006) and is expected to be at least 366 million in the year 2030 (Smyth and Heron, 2006). The International Diabetes Federation has declared that diabetes costs about 5-10% of the total healthcare budget in many countries (Smyth and Heron, 2006). The total cost of diabetes in the US according to the ADA was $132 billion in 2002 and this number is expected to rise to $156 billion in 2010 and to $192 billion in 2020 (Smyth and Heron, 2006).

**Metabolic Abnormalities**

Many serious and potentially life-threatening complications can develop in diabetic patients, and need careful attention and control. The most common of these complications are cardiovascular diseases (Haffner et al., 1998; Krone and Meinertz, 1995), blindness due to retinopathy, kidney diseases due to nephropathy, nervous system
diseases as a result of neuropathy, amputation, pregnancy complications, sexual dysfunction, and others. Since diabetes is associated with many complications, it is considered the sixth most common cause of death in the U.S. (Tsien et al., 1996).

**Glucose Homeostasis**

In the post-absorptive state (10-12 h overnight fast), the major fraction of glucose disposal occurs in the brain (50%), then 25% in the splanchnic area, which includes the liver and other gastrointestinal organs; the remaining 25% takes place in the insulin sensitive tissues in muscle and to a lesser extent in adipose tissue (Grill, 1990). Of the 25% peripheral glucose uptake, about 80-85% takes place in muscle and only 4-5% in adipose tissue (DeFronzo, 2004). However, this small fraction in adipose tissue is very important in glucose homeostasis, because it regulates adipose tissue release of FFA and adipokines that ultimately affect insulin sensitivity in liver and muscle (Bays et al., 2004; Bergman, 2000; Boden, 1997; Groop et al., 1989). After glucose ingestion, blood glucose level increases and stimulates insulin secretion. This hyperglycemia and hyperinsulinemia stimulates glucose uptake by splanchnic tissues as well as peripherals (primarily muscle) (DeFronzo, 1988). In addition, this will inhibit endogenous glucose production mainly in liver (hepatic glucose production [HGP]) (Cherrington, 1999; DeFronzo, 1988; DeFronzo and Ferrannini, 1987; DeFronzo et al., 1985; DeFronzo et al., 1981; Mari et al., 1994; Mittrakou et al., 1990). Insulin is known to have antilipolytic effects that inhibit the release of FFA from their triglyceride storage sites in adipose tissue. Even with small physiologic increases in plasma levels, there is reduction in
plasma FFA levels that will favor glucose uptake in muscle and inhibit HGP (Bays et al., 2004; Bergman, 2000; Boden, 1997; Groop et al., 1989). This is of great importance in normal physiological conditions in order to maintain normal glucose homeostasis.

Glucose homeostasis is under the control of glucagon, the level of which is important for the maintenance of normal hepatic glucose output and glucose concentrations. Glucagon secretion is inhibited by hyperinsulinemia after ingestion of glucose-containing meals in order to suppress HGP and maintain normal postprandial glucose tolerance.

Glucose homeostasis is greatly disturbed in T2DM patients. The changes are due to impaired insulin secretion, insulin resistance in insulin-sensitive tissues (liver, muscle, and adipose tissue), and impaired splanchnic glucose uptake (Cerasi, 1995; DeFronzo, 1988; Polonsky et al., 1996). Early in the development of T2DM, there is insulin resistance, although glucose tolerance remains normal. This is due to the fact that there is a compensatory insulin secretion from the β-cells to overcome this resistance. In the early stage of T2DM, β-cells also are still able to detect the severity of insulin resistance and adjust their secretion to keep normal glucose tolerance. It should be emphasized that T2DM starts with normal glucose tolerance, and insulin resistance with a compensatory insulin secretion. This will progress to impaired glucose tolerance and finally to T2DM. This was evident in many diabetic patients of different ethnic backgrounds; it was shown in Caucasians, Native Americans, Mexican Americans, and Pacific Islanders. It was evident in the rhesus monkey, which develops T2DM similar to humans (Bergman et al., 2002; Cerasi, 1995; DeFronzo, 1988; Haffner et al., 1995; Hansen and Bodkin, 1986; Kahn, 2001; Pimenta et al., 1995; Polonsky et al., 1996; Saad et al., 1988; Saad et al.,
A strong association between obesity and T2DM in these populations was observed, and because of this strong association, there is an emerging term “Diabesity” that describes this strong correlation (DeFronzo, 2004). In conclusion, it is important to stress that hyperinsulinemia precedes the development of T2DM and is an early sign of glucose intolerance and T2DM, and that overt diabetes does not develop before a significant decrease in $\beta$-cell ability to secrete physiological levels of insulin.

The progression of impaired insulin secretion implicates a number of factors including genetic and acquired factors. These factors are known to disturb the balance in “the state of dynamic change” in $\beta$-cells with respect to the continuous regeneration and apoptosis of the islets (Bonner-Weir, 2001). There is strong evidence that impaired $\beta$-cell function has genetic factors that are inherited (Barnett et al., 1981; Gautier et al., 2001; Vaag et al., 1995; Vauhkonen et al., 1998; Watanabe et al., 1999), and that impaired insulin secretion is an inherited trait, as was observed in Finnish families with T2DM (Watanabe et al., 1999). The acquired factors include, but are not limited to, glucotoxicity (DeFronzo, 1988; Rossetti et al., 1990) and lipotoxicity (Bays et al., 2004; McGarry, 2002; Shimabukuro et al., 1998; Unger, 1995). The negative effect of glucotoxicity on insulin secretion is supported by many studies. Improving glycemic control through diet, insulin therapy, or drugs has improved insulin secretion (Kosaka et al., 1980; Vague and Moulin, 1982).

Animal studies have shown that reduction in plasma glucose levels results in improved first- and second-phase insulin secretion. In these \textit{in vivo} studies, diabetic rats
were treated with a potent tubular glucose transporter inhibitor known as phlorizin, which reduces the plasma glucose levels (Rossetti et al., 1987). When phlorizin was administered to diabetic rats with partial pancreatectomy and chronic hyperglycemia, it restored normal glucose levels with improvement in first- and second-phase insulin secretion (Rossetti et al., 1987). This finding was supported by in vivo perfusion of glucose in non-diabetic rats with reduced β-cell mass, which showed about 75% decrease in insulin secretion (Leahy et al., 1988; Leahy et al., 1987). In addition, in vitro studies have shown that prolonged exposure of β-cells to high glucose concentrations downregulates insulin gene transcription and results in reduced synthesis and secretion (Robertson et al., 1994).

Another acquired factor that is implicated in β-cell dysfunction is lipotoxicity (Bays et al., 2004; McGarry, 2002; Prentki and Corkey, 1996; Shimabukuro et al., 1998; Unger, 1995). Lipotoxicity is caused by acute or chronic elevation in plasma FFA. Short-term physiologic elevation in plasma FFA causes an increase in insulin secretion. This happens via two mechanisms; the first is the conversion of FFA into fatty acyl-CoA derivatives in β-cells, which results in the increased production of phosphatidic acid and diacylglycerol (DAG). Both of these lipid intermediates will activate specific protein kinase C isoforms (PKC) that stimulate insulin exocytosis. The second mechanism is through fatty acyl-CoA, which stimulates exocytosis, causes closure of the ATP-sensitive K⁺ channels and stimulation of ATP-sensitive Ca²⁺ channels with subsequent elevation in intracellular calcium. All of these events cause increase in insulin secretion from the β-cells. On the other hand, chronic FFA elevation and exposure of β-cells lead to a
reduction in insulin secretion. This can occur due to indirect induction of nitric oxide synthase (iNOS) which in turn increases the expression of inflammatory cytokines like interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). These inflammatory cytokines are known to cause impairment of β-cell function and promote apoptosis (DeFronzo, 2004). Other factors in reduced β-cell function include: deficiency or resistance to incretins (Ahren et al., 1997; Creutzfeldt, 1979; D'Alessio et al., 1996; Drucker, 1998; Drucker, 2001; Holst et al., 1997; Nauck et al., 1993; Vilsboll et al., 2001), amylin (Islet Amyloid Polypeptide [IAPP]) (Howard, 1986; Johnson et al., 1989; Kahn et al., 1999), decreased number of β-cells in the pancreas (Butler et al., 2003; Clark et al., 1988; Gepts and Lecompte, 1981; Kloppel et al., 1985), and low birth weight due to poor nutrition and impaired fetal growth associated with low β-cell mass (Eriksson, 1996; Phillips, 1996).

**Insulin Action**

Insulin is a polypeptide hormone secreted from the β-cells in the pancreatic islets of Langerhans and was first isolated, purified and available for therapeutic application in 1921 (Wilcox, 2005). The name “insulin” was proposed by a Belgian scientist de Meyer in 1909, as well as a British investigator Schaefer in 1916 (Wilcox, 2005). It is a small globular protein of about 5.8 kDa in size, and consists of two chains connected by a disulfide bond. In β-cells insulin is first synthesized as a single chain of 86 amino acids known as preproinsulin, and then undergoes proteolytic cleavage producing another polypeptide called proinsulin. Proinsulin undergoes further cleavage to produce two
fragments; one with 31 amino acids known as C-peptide and the other, insulin, with two subunits (α and β, 21 and 30 amino acids length, respectively) (Figure 1).

Insulin synthesis and secretion are stimulated by glucose level of \( \geq 70 \text{ mg/dl} \) (3.9 mmol/L), which is the main regulator of insulin secretion by pancreatic β-cells. Insulin secretion is enhanced in response to increased levels of some amino acids, ketones, various nutrients, gastrointestinal peptides and neurotransmitters. Glucose regulates insulin on different levels: it enhances insulin translation, processing and induces secretion. The regulation process starts when glucose is transported via glucose transporters (GLUT2) into β-cells. Once in the β-cells, glucose undergoes phosphorylation via the rate-limiting enzyme in insulin secretion, glucokinase, which converts glucose into glucose-6-phosphate. It enters glycolysis to generate ATP and increase the ATP/ADP ratio. This will inhibit the activity of ATP-sensitive K⁺-channel that is composed of two components. The first component constitutes a receptor for binding the oral antidiabetic drugs sulfonylureas (SUR), and the second is a rectifying K⁺-channel protein. The inhibition of this channel induces a membrane depolarization of the β-cell membrane, which leads to the opening of a voltage-dependent calcium channel and ultimately the influx of calcium and subsequent secretion of insulin.
Figure 1. General Structure of Insulin
When secreted, insulin reaches the liver via the portal vein, where about 50% of the total secreted insulin will be degraded through the hepatic first pass effect. Un-degraded insulin reaches the systemic circulation and binds to insulin receptors in target tissues (DeFronzo, 1988; Pessin and Saltiel, 2000; Saltiel and Kahn, 2001; Whitehead et al., 2000). Insulin exerts many effects on target tissues: it decreases glucose production in liver and promotes fuel storage as glycogen in liver and muscle, and as triglycerides in adipose tissue. It also induces cell growth and proliferation.

Plasma insulin levels are regulated by three factors: (1) insulin synthesis and secretion from β-cells of the pancreas, (2) insulin sensitivity of the target tissues, and (3) insulin clearance. In case of T1DM, there is a loss of insulin secretion from the pancreas (absolute deficiency) due to an autoimmune disorder that impairs normal β-cell synthesis or secretion of insulin. The other main factor that regulates insulin levels is insulin sensitivity in target tissues. When there is insulin resistance in target tissues, the pancreas will respond to overcome this resistance by secreting more insulin. This leads to hyperinsulinemia (relative) as seen in insulin resistance states and T2DM. However, if the demand on insulin synthesis and secretion continues to increase, β-cell failure is the ultimate result, which leads finally to T2DM. Insulin levels are controlled by how much insulin is cleared mainly in the liver and to a lesser extent in the kidney. We have shown in our laboratory that insulin clearance is regulated by CEACAM1, which increases the rate of insulin endocytosis and degradation (Poy et al., 2002b).
After binding to its receptor, insulin leads to the activation of a cascade of phosphorylation-dephosphorylation reactions that finally give the different physiological functions of the hormone. Autophosphorylation of the insulin receptor (IR) is followed by further phosphorylation of downstream substrates including the insulin receptor substrates (IRSs), GAB-1, Shc, CEACAM1 (Najjar et al., 1995; Rees-Jones and Taylor, 1985), and many others. Phosphorylation of substrates engage, in turn, in the formation of signaling complexes via phosphotyrosine-containing binding motifs with Src homology-2 (SH2) domains found in molecules such as growth factor receptor binding protein (GRB-2), phosphatidylinositol (PI)-3’ kinase and others (Saltiel and Kahn, 2001; White, 1998). Phosphorylation of substrates and activation of downstream signaling molecules constitutes the basic mechanism of insulin action. Knockout mice have indicated that different substrates mediate the diverse effects of insulin (Accili et al., 2001; Kubota et al., 2000; Withers et al., 1998).

The Insulin Receptor (IR)

Insulin receptor is a transmembrane glycoprotein composed of two extracellular \( \alpha \)-subunits and two \( \beta \)-subunits that are connected by disulfide bonds (Figure 2). The \( \alpha \)-subunit is extracellular and has sites for insulin binding. The \( \beta \)-subunit consists of an extracellular domain, a transmembrane domain, and an intracellular domain that has the kinase activity (Accili et al., 2001) and is necessary for the phosphorylation and activation of downstream signaling molecules, as well as its own autophosphorylation.
Figure 2. Insulin Receptor Structure and activation

[Diagram showing the structure of insulin receptors, with inactive and active states, and key residues indicated.]
On the intracellular domain, IR has tyrosine residues (Y1158, 1163, and 1162) that work as major phosphorylation sites, and are required for the kinase activity of the protein (Chou et al., 1987; Ellis et al., 1986). The IR is encoded by the insulin receptor gene, which has 22 exons and is located on the short arm of human chromosome 19 (Ebina et al., 1985; Ullrich et al., 1985). Insulin receptor is first synthesized as a single polypeptide precursor that translocates into the plasma membrane to form a dimer after its aggregation with another monomer. The formation of this dimer produces the proreceptor which undergoes a proteolytic cleavage to produce the heterotetrameric insulin receptor (Alarcon et al., 1994). Insulin receptor is ubiquitously expressed almost in all tissues; however, it is widely present in the insulin target tissues: liver, skeletal muscle, and adipose tissue. It is in the pancreas (Harbeck et al., 1996), CNS (Marks et al., 1990), lymphatic cells (Berman and Center, 1987; Braciale et al., 1982) and the kidney (Arnqvist et al., 1988; Kurokawa et al., 1979).

**Insulin Signaling**

It is now known that all functions of insulin are mediated by the tyrosine kinase of the receptor and the downstream molecules. These findings were discovered after several studies that led to the cloning of the IR and its recognition as one of the tyrosine kinase family of receptors (Ebina et al., 1985; Ullrich et al., 1985). After binding to the $\alpha$-subunit of its receptor, insulin induces a conformational change in the extracellular $\alpha$-subunit; this in turn induces the autophosphorylation of the receptor and the activation of
the intrinsic kinase activity on the intracellular β–subunit. This autophosphorylation induces further phosphorylation of three main tyrosine residues (Tyr\(^{1146}\), Tyr\(^{1150}\), and Tyr\(^{1151}\)). This is followed by further phosphorylation of other tyrosine residues including Tyr\(^{960}\), which is located in the juxtamembrane domain, Tyr\(^{1316}\) and Tyr\(^{1322}\) in the C-terminus regulatory domain of the receptor. The phosphorylation of these residues is required for subsequent phosphorylation and activation of the IRS proteins (IRS-1, 2, 3, 4) (Lavan et al., 1997a; Lavan et al., 1997b; Patti et al., 1995; Sun et al., 1991; Yenush and White, 1997) and Shc (Pronk et al., 1993). Eventually, these phosphorylation cascades create docking sites for downstream binding proteins with diverse functions. The activation of multiple protein mediators accounts for the different functions of insulin. Two major pathways that are activated by insulin which ultimately provide these actions: the PI-3 kinase pathway and the Ras/Raf/MAP kinase pathway (Figure 3). In the earlier, insulin regulates several functions including: glucose transport, glycogen synthesis, protein synthesis, anti-lipolysis, anti-apoptotic, and regulates gene expression and mitogenesis. Through the latter pathway, insulin works mainly on gene expression and mitogenesis (Le Roith and Zick, 2001).

**PI3K Pathway**

The PI-3 kinase consists of two subunits; a p85 regulatory subunit and a p110 catalytic subunit. After phosphorylation by the tyrosine kinase of the IR, the regulatory subunit of PI-3 kinase, which has phosphotyrosine-binding SH2 domain, will activate the p110 catalytic subunit (Backer et al., 1992; Myers et al., 1992). This activation is
Figure 3. Insulin Signaling Pathways and Main Functions

- **Glucose**
  - **Insulin**
  - **pY960**
  - **IRS**
  - **p85, p110**
  - **PI3K**
  - **Akt**
  - **14-3-3, BAD**
  - **Anti-apoptosis**
  - **Glycogen synthesis**
  - **Protein synthesis**
  - **Fatty acid synthesis**
  - **Gene transcription**
  - **MAPK**
  - **Mitogenesis**

- **Glucose**
  - **Insulin**
  - **pY960**
  - **IRS**
  - **p85, p110**
  - **PI3K**
  - **Akt**
  - **14-3-3, BAD**
  - **Anti-apoptosis**
  - **Glycogen synthesis**
  - **Protein synthesis**
  - **Fatty acid synthesis**
  - **Gene transcription**
  - **MAPK**
  - **Mitogenesis**
important in order to bring the PI-3 kinase closer to the membrane bound substrates. As a result of PI-3 kinase activation, there is an increase in the production of phosphatidylinositol (3,4,5) triphosphate (PIP$_3$), which in turn binds to the pleckstrin homology (PH) domains of a variety of signaling molecules. The signaling molecules that are included in this activation are the phosphinositide-dependent kinase 1 (PDK1) and protein kinase B (Akt/PKB). In addition, Akt/PKB and the atypical protein kinase C (PKC) isoforms $\zeta$ and $\lambda$ are also important to augment the PI-3 kinase-mediated Glut4 translocation in response to insulin activation (Bandyopadhyay et al., 1997; Kohn et al., 1996; Standaert et al., 1997). Due to this activation, these proteins will change their activity and subcellular activation, which in turn leads to the different downstream effects (Alessi et al., 1997; Lietzke et al., 2000).

PI-3 kinase pathway is one of two parallel pathways in the insulin-mediated glucose uptake by many tissues especially the muscle and fat cells. Although the PI-3 kinase constitutes the major pathway in glucose uptake in response to insulin activation via glucose transporter-4 (Glut4) in particular in muscle and adipose tissue (Haney et al., 1995; Jhun et al., 1992); however, it is not sufficient for the translocation of Glut4 in response to insulin (Jiang et al., 1998; Sharma et al., 1997). In addition to PI-3 kinase, there is an alternative pathway that recruits Cbl to the IR via c-Cbl-associated protein (CAP). Once Cbl is associated to the IR, it undergoes phosphorylation followed by translocation of CAP-Cbl-flotillin complex to the lipid raft domain (Baumann et al., 2000). This is followed by compartmentalization of CAP-Cbl in lipid rafts where Cbl
activates the small GTP-binding protein TC10, which leads to cytoskeletal rearrangements and finally to enhanced Glut4 localization at the membrane independent of the PI-3 kinase pathway (Chiang et al., 2001).

In addition to its major function in glucose transport, Akt/PKB has many other functions: it is important in the regulation of glycogen and protein synthesis, promotion of cellular growth (anti-apoptotic action) (Saltiel and Pessin, 2002; White and Kahn, 1994) and others. In response to insulin activation, Akt inactivates glycogen synthase kinase 3 (GSK 3) by phosphorylation, which in turn activates the rate-limiting enzyme in glycogen synthesis, the glycogen synthase (GS), which promotes glycogen synthesis (Cross et al., 1995). Akt/PKB is involved in protein synthesis through inactivation of GSK 3, which leads to the activation of translational initiation factor eIF2B, which promotes protein synthesis in response to insulin activation (Welsh et al., 1998; Welsh and Proud, 1993). Akt/PKB mediates the antiapoptotic effect of insulin. This can be achieved by phosphorylation of BAD and Caspase 9, which in turn inhibits the apoptotic machinery (Datta et al., 1997). Another mechanism by which Akt regulates cellular growth is through its ability to phosphorylate the forkhead gene family transcription factor (FKHRL1). This activates FKHRL1 and leads to its association with 14-3-3 proteins and localization to the cytoplasm. As a result of FKHRL1 activation, there is a decrease in the expression of genes important in cell death such as the Fas ligand gene (Brunet et al., 1999; Nakae et al., 1999).
**Ras/MAP Kinase Pathway**

The other major pathway that is activated in response to insulin is the Ras/MAPK pathway. The activation of this pathway takes place after activation of IRS proteins and Shc and through their association via the adaptor protein Grb2 (Myers et al., 1994; Skolnik et al., 1993). The activated MAPK forms a dimer and translocates into the nucleus where it phosphorylates transcription factors such as Elk-1 (Cobb, 1999) and p62TCF (Boulton et al., 1991). The activation of the transcription factors leads to increased gene transcription and insulin-induced gene expression, cell proliferation and differentiation (White, 1997; White, 2002).

**Insulin Receptor Substrates**

Insulin receptor is a member of the tyrosine kinase (TK) family of receptors that recruits downstream protein substrates in order to deliver the signals and elicit the different functions of insulin. Among the most important substrates are the IRSs (IRS-1, -2, -3, -4) (Lavan et al., 1997a; Lavan et al., 1997b; Patti et al., 1995; Sun et al., 1991; Sun et al., 1995; Yenush and White, 1997), Shc (Koch et al., 1991), and CEACAM1 (Najjar et al., 1995).

**The IR Substrates (IRS) Proteins**

So far, four members of the IRS family are known: IRS-1, -2, -3, and -4. They show the highest shared homology at the N-terminal PH domain, followed by the adjacent PTB domain. This PTB domain is important for binding of the IRS to IR
through the NPXY$^{960}$ motif on IR after phosphorylation. These protein substrates have 8-18 potential tyrosine phosphorylation sites that form docking sites for the SH2-containing proteins downstream of IRS proteins such as Grb2, Src-homology Phosphatase-2 (SHP2), and PI-3 kinase (Backer et al., 1992; Myers et al., 1992). Because there are many downstream proteins that can interact with IRS proteins, insulin affects the body in many ways (White, 2002). Of the four known members of the IRS family, IRS-1 and –2, which are ubiquitously expressed, are the major mediators of insulin signaling and their functions are complementary. However, IRS-1 is the most characterized IRS and is more highly expressed than IRS-2 in muscle and adipose tissue (Araki et al., 1993; Sun et al., 1995). The main function of IRS-1 is its role in the regulation of mitogenic pathways. On the other hand, IRS-2, which is predominantly in the liver and is more largely expressed than IRS-1 (Araki et al., 1993; Sun et al., 1995), is the main mediator of the metabolic effects of insulin and hepatic glucose homeostasis. IRS-3 is widely expressed in adipose tissue (Lavan et al., 1997b) while IRS-4 is largely expressed in the pituitary, thymus, and brain (Uchida et al., 2000).

In order to further elucidate the functions of the different IRS proteins and their metabolic roles in insulin action, researchers have used gene knockout techniques in murine models as a tool to further understand their roles. When they deleted the IRS-1 gene ($Irs^{-/-}$) in mouse, hyperinsulinemia and insulin resistance developed predominantly in muscle. However, due to a compensatory β-cell hyperplasia, mice were protected from developing diabetes (Araki et al., 1994; Tamemoto et al., 1994). This supports the notion that IRS-1 regulates glucose uptake in muscle and adipose tissue and
has a major effect on somatic growth. Further studies carried out on IRS-2 by gene knockout (\(Irs-2^{-/-}\)) in mice have shown the development of T2DM within 10 wk. The mice exhibited plasma random hyperglycemia, hyperinsulinemia, glucose intolerance and severe insulin resistance in liver and muscle (Withers et al., 1998). Moreover, they also developed abnormal glucose transport in peripheral tissues in response to insulin activation, in addition to unsuppressed insulin-induced hepatic glucose production and output, and lipolysis (Withers and White, 2000). At later stages, hypoinsulinemia will develop due to \(\beta\)-cell mass reduction and failure. When IRS-3 and IRS-4 genes were deleted, no major abnormalities occurred in mice; however, there was a slight decrease of body weight, mild fasting hypoglycemia and mild reduced infertility (Fantin et al., 2000).

**Shc**

Another key player in insulin signaling is Shc which is one of the main substrates of the tyrosine kinase family of receptors. In addition to its activation via the IR tyrosine kinase, Shc is activated by the tyrosine kinases of IGF-I, platelet derived growth factor (PDGF) receptor, fibroblast growth factor (FGF) receptor, and epidermal growth factor (EGF) receptor (Pellicci et al., 1992; Pronk et al., 1994; Rozakis-Adcock et al., 1992; Yokote et al., 1994).

There are three Shc genes that have been cloned in mammal: ShcA, which is ubiquitously expressed, ShcB and ShcC which are limited to neuronal cells (Pellicci et al., 1996). The protein product of all Shc isoforms have the same structure which consists of a C-terminal SH2 domain connected to a collagen homology (CH) domain that is rich in
glycine/proline residues. On the N-terminal, it is connected to a PTB domain (Migliaccio et al., 1997). The physiologic importance of Shc was studied via gene knockout technology in mice, in which the deletion of Shc causes defects in the cardiovascular system development and death of pups between embryonic days 9 and 12 (Lai and Pawson, 2000). Two major roles for Shc in signaling: a role in the activation of ras/MAP kinase pathway to regulate cell growth, and a role in receptor-mediated protein endocytosis. In response to TK activation upon ligand binding and autophosphorylation, Shc interacts via its SH2 domain (Pelicci et al., 1992) or PTB domain (Blaikie et al., 1994; Kavanaugh and Williams, 1994) with the receptor’s phosphotyrosine. This leads to the phosphorylation of Shc and the formation of a complex with Grb2 (Rozakis-Adcock et al., 1992). This will lead to the association of Shc/Grb2 with the guanine nucleotide exchange factor Sos, and translocation close to the membrane where the complex will activate the member-bound inactive Ras-GDP into the active form Ras-GTP. This, in turn, will activate Raf, MEK, and MAPK kinases (Sasaoka et al., 1994). The other function of Shc is to target proteins to clathrin-coated pits in receptor-mediated endocytosis processes. ShcA has several conserved sequences in the area a.a. residues 346-355 (RDLFDMKPFEE) that provides a binding site to the clathrin-coated pit adaptor complex (Okabayashi et al., 1996). Shc has two aromatic residues (Phe349 and Phe354) that are required for rapid internalization of EGFR (Sakaguchi et al., 2001). Internalization of EGFR occurs after EGF activation of the receptor, followed by association of Shc with the α-chain of AP2 complex. This complex will mediate the EGF-mediated internalization of the EGFR. In a similar way, Shc uses its PTB domain to bind the IR on
Tyr\textsuperscript{960} in the juxtamembrane domain (Sasaoka et al., 1994). On the other hand, Shc uses its SH2 domain to bind CEACAM1-4L (Poy et al., 2002a), by doing so, Shc mediates the indirect interaction between IR and one of its TK substrates CEACAM1-4L. In this manner, Shc is involved in CEACAM1-4L-mediated increase in the rate of receptor-mediated insulin endocytosis in cultured hepatocytes and mouse liver (Formisano et al., 1995; Najjar et al., 1998; Poy et al., 2002b).

Overexpression of SH2 domain of Shc and Grb2 inhibits the interaction between CEACAM1 and Shc as well as the interaction between Grb2 ad Shc. In case of CEACAM1/Shc inhibition of interaction, the mitogenic effect of insulin was increased and insulin endocytosis was decreased. On the other hand, inhibiting Grb2/Shc interaction has lead to downregulation of mitogenesis and increased receptor endocytosis. Taken together, this shows a dual role for the complex CEACAM1/Shc in insulin signaling via two suggested mechanisms: in the first, CEACAM1 sequesters Shc and directs it preferentially towards the endocytosis pathway. The net result of this binding is increased rate of insulin internalization and downregulation of insulin signaling. The second mechanism is by sequestering Shc; CEACAM1 will divert Shc from the mitogenic pathway, as a result decreasing the mitogenic effect of insulin.

**CEACAM1**

CEACAM1 is a substrate of the IR tyrosine kinase in liver. It is a highly glycosylated transmembrane glycoprotein that was originally identified in rat hepatocytes (Rees-Jones and Taylor, 1985). CEACAM1, was known as pp120 and biliary
glycoprotein (BGP) (Beauchemin et al., 1999), is ubiquitously expressed with wide expression in the liver, but not in muscle or adipose tissue (Accili et al., 1986). The transcript of CEACAM1, but not the protein product, was detected in muscle (Mittelman et al., 2000; Randle et al., 1963). CEACAM1 is synthesized in endothelial cells, B and interleukin-activated T cells, and in epithelial cells of the gastrointestinal tract, breast, prostate and endometrium (Sadekova et al., 2000; Wagener and Ergun, 2000).

Rat CEACAM1-4L (referred to as CEACAM1) is a transmembrane glycoprotein that is highly conserved among the different species. Its predicted amino acid sequence in rat is 519 a.a. long (Figure 4). The extracellular domain of CEACAM1 contains 4 IgG-like structures (hence, 4 in front of L in CEACAM1-4L), with the N-terminal V-like domain containing a salt bridge, and three C2 domains (A1, B1 and A2). It also contains several N-linked glycosylation sites (Obrink, 1997). The 71 amino acid-long intracellular domain includes serine (Ser503) and tyrosine (DDVxTyr488) phosphorylation sites (-p in Figure 4).

**CEACAM1 structure and gene organization**

CEACAM1 gene has nine exons. Alternative splicing of the 7th exon during mRNA processing yields two isoforms that differ by the presence (-4L) or absence (-4S) of most of the intracellular domain, including the phosphorylation sites. Ceacam1 gene (Cc1) is housekeeping (it lacks a functional TATA box and contains multiple Sp1 binding sites and transcription initiation sites at nucleotides -101, -71, -41, and -27 spread over a GC-rich region) and contains a functional promoter and nine exons (Najjar et al.,
1993; Najjar et al., 1996). The Sp1 boxes 3 (nt-157 and -149) and 5/6 (nt-131 to -117) are required for basal transcription of the Cc1 gene.

Figure 4. Structure of CEACAM1-4S and CEACAM1-4L
Several studies have shown that insulin, dexamethasone, and cAMP increase the promoter activity of rat Cc1 gene in transfected rat H4-II-E hepatoma cells by 2- to 3-fold (Najjar et al., 1993; Najjar et al., 1996). Moreover, the promoter activity was increased 4- to 5-fold after phorbol ester and cAMP stimulation together; however, it was unaffected by phorbol esters alone.

**Regulation of CEACAM1 Phosphorylation**

In response to insulin stimulation, the long isoform (-4L) but not the short isoform of CEACAM1 (-4S) undergoes phosphorylation by the IR (Najjar et al., 1995) and EGFR (Abou-Rjaily et al., 2004). What makes CEACAM1 different from other IR substrates is the fact that it is not phosphorylated by the IGF-1 receptor tyrosine kinase in response to insulin stimulation (Soni et al., 2000). In addition to this, its phosphorylation requires the non-conserved Tyr\textsuperscript{1316} in the C-terminus of the \(\beta\)-subunit of IR, as opposed to other substrates that require a conserved Tyr\textsuperscript{960} in the juxtamembrane domain of the receptor (White, 1998). CEACAM1 phosphorylation on Ser\textsuperscript{503} takes place in the absence of insulin (Estrera et al., 2001; Najjar et al., 1995) and EGF (Abou-Rjaily et al., 2004). It has been shown that this phosphorylation is required for tyrosine phosphorylation on Tyr\textsuperscript{488} in order to activate CEACAM1 and elicit the phosphorylation-dependent functions. However, what phosphorylates Ser\textsuperscript{503} is still to be elucidated. Ser\textsuperscript{503} phosphorylation can be induced by the activation of PKC using the phorbol ester PMA, but the phosphorylation was not inhibited when PKC was inhibited by calphostin C (Fournes et
al., 2001). Tyr$^{513}$ is not phosphorylated by insulin or EGF; however, an intact Tyr$^{513}$ is required for phosphorylated CEACAM1 to activate a serine phosphatase in response to insulin (Najjar et al., 1998). In addition to the above regulators of CEACAM1, its phosphorylation is tightly regulated via phosphatases that are associated with the protein as SHP-1 and SHP-2 (Huber et al., 1999).

**CEACAM1 Functions**

A lot of research work has to be done to elucidate the functions of CEACAM1. Of the many functions that CEACAM1 has, it is known to act as cell adhesion molecule that mediates Ca$^{2+}$-independent homophilic and, to some extent, heterophilic, cell-adhesion (Obrink, 1997). In addition, it may suppress tumor growth (Carter and Sorkin, 1998), it has a role in mediating angiogenesis (Ergun et al., 2000), and a role in mediating the inflammatory response to Neisseria infection (Muenzner et al., 2001). In respect to insulin action, CEACAM1 has been shown in our laboratory to downregulate the mitogenic effect insulin (Formisano et al., 1995; Soni et al., 2000) and EGF (Abou-Rjaily et al., 2004), and mediates insulin clearance in liver (Poy et al., 2002b) through its upregulation of receptor-mediated insulin endocytosis and degradation (Choice et al., 1998; Formisano et al., 1995; Li Calzi et al., 1997). Our laboratory has recently shown that CEACAM1 interacts with FAS to tightly regulate fat metabolism and preserve insulin sensitivity in liver (Najjar et al., 2005). It achieves this effect by mediating the acute effect of insulin on FAS activity. We have shown that CEACAM1 appears to be required for cells to respond intrinsically to acute changes of insulin levels, and that the
acute effect of insulin on FAS activity depends on CEACAM1 phosphorylation rather than FAS content (Najjar et al., 2005). With the exception of cell adhesion, all of these functions require CEACAM1 to be primarily phosphorylated on its intracellular tail (Carter and Sorkin, 1998; Formisano et al., 1995; Soni et al., 2000).

**Regulation of Insulin Action by CEACAM1**

Insulin clearance is necessary for the normal functioning of the hormone. It is defined as the plasma volume being cleared of insulin in a unit of time (Valera Mora et al., 2003). Insulin clearance occurs in many organs and cells. Liver is the most important organ, followed by the kidneys, adipose, muscle, fibroblasts, gastrointestinal cells and others. For insulin clearance to occur, insulin should bind to its receptor, internalized as insulin-insulin receptor complex and degraded by the insulin degrading enzyme (IDE), insulinase or lysosomal enzymatic processes (Valera Mora et al., 2003). A major part of insulin is internalized by receptor-mediated process (Carpentier et al., 1993). It also can take place by pinocytosis in cases of hyperinsulinemia (Duckworth et al., 1998).

CEACAM1 is phosphorylated by IR in response to insulin activation (Najjar et al., 1995). It undergoes phosphorylation in absence of insulin on Ser^{503} (Kharitonenkov et al., 1997), which is needed for the insulin induced phosphorylation on Tyr^{488} in hepatocytes on its intracellular domain (Choice et al., 1998; Najjar et al., 1998). CEACAM1 increases the rate of receptor-mediated insulin endocytosis and degradation (Formisano et al., 1995; Li Calzi et al., 1997) to mediate insulin clearance in liver (Poy et al., 2002b). It binds indirectly to the receptor to undergo internalization in clathrin-coated vesicles as of the
insulin endocytosis complex (Poy et al., 2002b). This binding in response to CEACAM1 phosphorylation increases the rate of internalization and degradation of insulin. However, receptors recycle and return back to the plasma membrane. We developed an animal model in our laboratory that has a liver specific S503A CEACAM1 (L-SACC1) transgenic mouse, with functional inactivation of Ceacam1 gene, carrying a dominant-negative phosphorylation-defective rat S503A Cecam1 mutant in liver (Poy et al., 2002b). Inactivation of CEACAM1 produced a characteristic animal model of impaired insulin clearance, elevated FFA and triglycerides, visceral adiposity, normal fasting but high random blood glucose, glucose intolerance and insulin resistance (Poy et al., 2002b). This animal model made it easy to understand the function of CEACAM1 protein in insulin clearance.

The amount of circulating insulin in the blood stream is an important factor that regulates insulin action. This is in turn determined by how much insulin is secreted from the pancreatic β-cells and insulin clearance, a process that mainly takes place in liver and to a lesser extent in kidney (Duckworth et al., 1998). When CEACAM-4L, but not -4S and the site-directed phosphorylation-defective Ser503 to Ala (S503A) and Tyr488 to Phe (Y488F) mutants of CEACAM1, is co-expressed, it increases insulin endocytosis and degradation in NIH 3T3 cells in a receptor-mediated manner (Formisano et al., 1995; Najjar et al., 1998). CEACAM1 promotes insulin internalization by IR-A (an isoform of IR with high-affinity that is predominantly expressed in liver) and targets insulin for degradation. In hyperinsulinemia CEACAM1 increases that rate of insulin removal via IR-A, in this manner preventing downregulation of the high-affinity receptors. This may
explain why IR in liver is less sensitive to insulin-induced receptor downregulation. Additionally, this provides a mechanism by which liver protects itself against the physiologically high insulin levels in the portal circulation that usually has 2- to 3-fold higher insulin than the systemic circulation. Our laboratory has shown that CEACAM1 should be phosphorylated and that IR should have intact Tyr$^{960}$ in its juxtamembrane domain in order for CEACAM1 to increase the IR-induced insulin endocytosis (Najjar et al., 1998). Although Tyr$^{960}$ is required for CEACAM1 action to promote IR endocytosis, it is not enough to do so. This finding came after the failure of CEACAM1 to regulate IGF-1 endocytosis by its receptor which also has the conserved Tyr$^{960}$ (Najjar et al., 1998). This made Najjar and co-workers propose that another non-conserved Tyr residue (Tyr$^{1316}$), which is present at the C-terminus of the β-subunit of IR, is necessary to phosphorylate CEACAM1 on Tyr$^{488}$ (Najjar et al., 1998). This makes CEACAM1 as a specific substrate of IR and distinguishes it from its close relative IGF-1 receptor, in which Tyr$^{960}$ is conserved. In this manner, CEACAM1 has a specific role for IR endocytosis recruiting Tyr$^{960}$ in IR in the formation of insulin-receptor endocytosis complex (Figure 5). These studies are supported by further in vivo studies at our laboratory using liver-specific overexpression of the dominant negative phosphorylation-defective S503 CEACAM1 (L-SACC1) mutant. The resultant mouse developed hyperinsulinemia due to impairment of insulin clearance, visceral adiposity, and elevated plasma FFA and triglyceride levels (Poy et al., 2002b). The fact that L-SACC1 mice with the inactive CEACAM1 developed hyperinsulinemia is consistent with the hypothesis that CEACAM1 upregulates insulin degradation in liver. As result of hyperinsulinemia
Figure 5. The Insulin Receptor Endocytosis Complex
secondary insulin resistance developed (Park et al., 2006) along with random, but not fasting, hyperglycemia. In support of this work, when the phosphatase of CEACAM1 (SHP-1) was activated, it negatively affected glucose homeostasis due to impairment of insulin clearance (Dubois et al., 2006). Taken together, these data suggest that CEACAM1 comprises a novel mechanism in regulating insulin sensitivity through its effect on insulin clearance in liver.

**CEACAM1 Decreases Insulin-mediated Hepatic de novo Lipogenesis**

**Fatty Acid Synthase (FAS)**

Fatty acid synthase (FAS) is a key enzyme in the de novo synthesis of fatty acids. It is highly expressed in liver and to a lower extent in white adipose tissue (WAT) (Cantley, 2002). The main function of FAS is to activate the conversion of malonyl-CoA to the saturated fatty acid palmitate. Malonyl-CoA works as a fuel sensor that inhibits carnitine palmitoyl transferase-1 (CPT-1) a key enzyme that catalyzes the transport of long chain fatty acids (LCFA) to mitochondria for oxidation. Another function of FAS has been recently found; it induces fatty acid oxidation by providing fatty acid products that work as natural ligands of PPARα (Chakravarthy et al., 2005).

**FAS and Insulin Action**

Insulin regulates fatty acid synthesis by increasing mRNA transcription of FAS. This is observed in chronic elevation of plasma insulin such as in obesity (Zucker [fa/fa] rats (Bazin and Lavau, 1982) and JCR: LA-corpulent [Cp/Cp] obese rats (Shillabeer et al.,
1992)) and in hyperinsulinemia, in which FAS mRNA transcription is increased as well as other lipogenic enzymes known to increase lipogenesis. The FAS plays an important role in the pathogenesis of obesity as observed by reduction of body weight of obese mice treated with FAS inhibitors that regulate the levels of malonyl-CoA independently of leptin (Loftus et al., 2000). Activation of hepatic de novo fatty acid synthesis negatively affects insulin action and increases body weight. These effects were supported by increasing the degradation of malonyl-CoA by overexpressing the enzyme malonyl-CoA carboxylase, which in turn decreases the availability of malonyl-CoA as a substrate for FAS (An et al., 2004). In accordance with this, when malonyl-CoA was inhibited, insulin sensitivity was improved and body weight was decreased (An et al., 2004).

As compared to its chronic positive effect on FAS, we have shown in our laboratory that insulin acutely decreases FAS activity in liver, but not in adipose tissue (Najjar et al., 2005). Inducing insulin surges in mice after 1, 4 and 7 h of refeeding decreases FAS activity in liver. This decrease in FAS activity depends on two factors: the first factor is the ability of insulin to phosphorylate CEACAM1, internalization of CEACAM1 in the insulin endocytosis complex (Najjar et al., 1998) and binding to FAS. They second factor is the prior insulnemic state (Najjar et al., 2005) as observed in L-SACC1 transgenic mice and Ob/Ob, which have chronic hyperinsulinemia, however, insulin did not decrease FAS activity in these animals. The reason for this is that insulin becomes unable to activate its receptor and that this impairment in IR activation (phosphorylation) will be reflected on its ability to phosphorylate CEACAM1 in chronic
hyperinsulinemia (Najjar et al., 2005). This constitutes a mechanism by which liver limits possible lipogenesis that could develop due to chronic hyperinsulinemia in portal circulation (Ward et al., 1990). This decrease in lipogenesis occurs due to a decrease in FAS activity in liver, as a result of acute negative effect of insulin on CEACAM1 which is mainly found in liver, and not in WAT. The consequence of this acute downregulation of FAS activity by insulin is reflected on triglycerides synthesis and output from liver. Taken together, this stresses a dual action for CEACAM1 to promote insulin clearance and reduce lipogenesis in liver putting it as an important player in the pathogenesis of obesity and T2DM.

**CEACAM1 Role in Insulin and Fat Metabolism**

CEACAM1 shows a key role in maintaining insulin action by promoting insulin clearance which affects lipid in liver as our laboratory has previously shown in the L-SACC1 mouse phenotype (Dai et al., 2004). As a results of impaired insulin clearance, hyperinsulinemia develops with insulin resistance in liver as shown by insulin-induced suppression of gluconeogenesis in hyperinsulinemic-euglycemic clamp studies (Park et al., 2006), and elevated *de novo* triglyceride synthesis and secretion as very low-density lipoproteins (VLDL). This in turn will stimulate β-cells to secrete more insulin to compensate for decreased insulin sensitivity and to sensitize peripheral tissues (adipose tissue and muscle) towards triglyceride and increase its uptake. This in turn will increase adipocyte proliferation and the mass of the adipose tissue with decrease glucose uptake into muscle and adipose tissue.
**Insulin Resistance**

Insulin resistance is the reduced ability of insulin to work effectively on peripheral insulin target tissues (in particular muscle, adipose tissue, and liver). This decreased ability is caused by a combination of genetic susceptibility and obesity and its complications. Its resistance involves inadequate cellular response to normal circulating plasma insulin levels. Therefore, extra amounts of insulin should be secreted to compensate for resistance and in order to maintain normal blood glucose levels (Eckel et al., 2005; Wang et al., 2004). Insulin resistance is characterized by a compensatory hyperinsulinemia, inability to stop lipolysis from adipose tissue, unsuppressed gluconeogenesis, and reduced glucose uptake in skeletal muscle. Insulin resistance is the hallmark of T2DM with an overall prevalence of 10-25% (Ferrannini et al., 1997). It is also linked to many diseases including obesity, T2DM, metabolic syndrome, lipodystrophies, polycystic ovary syndrome and chronic infection (Mlinar et al., 2007).

Insulin resistance develops as a result of the interaction between genetic factors and environmental factors such as food intake, lack of exercise, smoking, aging, certain drug intake like thiazide diuretics, beta blockers, glucocorticoids, and others (Granberry and Fonseca, 1999). Much of research has been done on obesity and its role in the development of insulin resistance; it is considered one of the most important diseases associated with insulin resistance as it also results from a combination of genetic and environmental factors (Cummings and Schwartz, 2003; Granberry and Fonseca, 1999). Due to insulin resistance in obesity, FFA will be released from visceral adipose tissue because of the inability of insulin to inhibit lipolysis. As a result of excessive FFA release,
insulin resistance gets worse because FFA contributes to further insulin resistance. Moreover, visceral adipose tissue also secretes other hormones and adipokines that regulate insulin action and contributes to further insulin resistance. The fact that visceral obesity increases insulin resistance is supported by improvement of insulin sensitivity after caloric restriction, weight loss and increased physical activity (Granberry and Fonseca, 1999; Greenfield and Campbell, 2004; Grundy et al., 2004).

The cause of insulin resistance is not clear yet, and still under debate. However, it is strongly believed that chronic FFA elevation impairs insulin signaling pathways and causes insulin resistance (Carmena, 2005) and may be due to any of three general mechanisms: (1) an abnormal insulin molecule, (2) an excessive amount of circulating antagonist, and (3) target tissue defects, with the last is most common cause of insulin resistance. There is a strong correlation between insulin resistance and central body part obesity (Visceral obesity), high-fat intake and lack of exercise. The three conditions share elevation of FFA; however, visceral fat is the main contributor to high FFA flux which in turn leads to insulin resistance. Visceral adipose tissue also releases hormones and cytokines (like tumor necrosis factor-alpha (TNF-α), IL-6 and others) that potentiate insulin resistance (Carmena, 2005).
Abnormal Insulin Signaling

Impaired insulin action can take place at three possible levels: (1) at the pre-receptor which includes inactivation of insulin while circulating in the blood stream, (2) at the receptor level due to any defects in IR, and (3) at the post-receptor level. What is common between these three levels is that they are all caused by either genetic or environmental factors, however, very few of insulin resistance cases are caused by a single genetic or acquired trait (Mlinar et al., 2007). Insulin resistance can develop at the pre-receptor level as in type 1 diabetes when autoantibodies are developed against insulin leading to the degradation of the hormone (Sharma et al., 1991). It can also develop at the receptor level as in the IR mutations that develop in the IR gene as seen in type A insulin resistance which is responsible for reduced Tyrosine phosphorylation of the β-subunit after insulin binding due to a heterozygous genetic mutation (McIntyre and Walker, 2002). It may also develop as a result of reduced insulin binding to its receptor as in Rabson-Mendenhall syndrome and leprechaunism (Donohue syndrome) (McIntyre and Walker, 2002) as well as the formation of antibodies against IR itself (type B insulin resistance) (O'Rahilly, 2002). Another factor in developing insulin resistance at the receptor level is the degradation of the IR (Taylor et al., 1982) such as in ligand-induced insulin receptor downregulation. Moreover, the reduced activity of the receptor also plays a role in development of insulin resistance as suggested by phosphorylation of IR on Ser/Thr residues by different PKCs (Bossenmaier et al., 2000) that ultimately decreases phosphorylation on Tyrosine residues in IRS-1 (Saad et al., 1992). The post-receptor events received more investigation as they include many signaling molecules downstream
of IR. IRS-1 is a major target of studies dealing with insulin resistance. Many factors can increase Ser/Thr phosphorylation of IRS-1 including TNF-α, hyperinsulinemia secondary to insulin resistance, PKCs, PI3-K downstream kinases, MAP kinases (Erk1 and Erk2) (Mlinar et al., 2007). This in turn leads to a subsequent increase in proteosomal degradation of IRS-1 (Pirola et al., 2004). Although some basal Ser/Thr phosphorylation is required for the normal phosphorylation on Tyr, it is still unclear on which site on IRS-1 this Ser/Thr takes place and as well as to what degree it should happen (Mlinar et al., 2007). The amount of IRS-1 also plays an important role in the development of insulin resistance as noticed in human subjects, animals and tissue culture cells with insulin resistance (Pirola et al., 2004). Another post-receptor signaling molecule that is involved in development of insulin resistance is IRS-2, which is downregulated by hyperinsulinemia and negatively changes downstream insulin signaling (Pirola et al., 2004).

The adipose tissue-derived effectors (such as FFA and adipokines) play an important role in the development of insulin resistance. The effects of these effectors will be discussed in details in a later section. The suppressors of cytokine signaling-1 and -3 (SOCS-1 and -3) are a group of proteins that play an important role in insulin signaling and development of insulin resistance. These proteins are usually activated by TNF-α and IL-6 and can negatively affect insulin action through their association with IR in competition with IRS-1, inhibiting Janus kinase, and by helping proteosomal degradation of IRS-1 (Kile et al., 2002). In addition, increased FFA release from adipose tissue increases plasma FFA levels which after metabolism produce lipid intermediates such as
diacylglycerol and acyl-CoA that increase Ser phosphorylation on IRS-1 by PKC-θ. They also decrease activity of several signaling molecules including IRS-1, IRS-2, PI3 kinase, PKB-α, PKC-λ, PKC-ζ, and GSK in rat muscle (Pirola et al., 2004).

**Knockout and Transgenic Animal Models of Insulin Resistance**

Since insulin signaling involves several signaling molecules and pathways to elicit the different functions of insulin, researchers have manipulated different knockout models to study these signaling molecules and their role in insulin action. Some of these mouse models were modified by modifying two genes in insulin signaling pathways in an attempt to simulate the polygenic nature of T2DM (Plum et al., 2005).

*Insulin receptor knockouts:* heterozygous IR knockout mice indicated that 50% of IR is sufficient to maintain normal blood glucose levels. On the other hand, homozygous IR knockout mice died after 3-7 d after birth because of diabetic ketoacidosis (Accili et al., 1996; Joshi et al., 1996).

*Muscle specific insulin receptor knockout (MIRKO) mice:* showed altered fat metabolism including accumulation of fat, elevated serum triglyceride and FFA levels (Bruning et al., 1998). Moreover, it has been shown that MIRKO mice showed redistribution of substrates to adipose tissue, which contributes to increased adiposity in these mice (Kim et al., 2001). Although this specific muscle IR deficiency caused muscle-specific insulin resistance; blood glucose, serum insulin and glucose tolerance were normal in these mice indicating that other tissues other than muscle are more important for insulin-stimulated glucose disposal. Specific muscle glucose transporter 4
GLUT4 deficiency leads to impaired insulin stimulated glucose transport into muscle cells (Zisman et al., 2000). GLUT4 translocates in response to insulin activation of the IR, interestingly it also responds to the closely related hormone IGF-1 activation of the IGF-1R in a compensatory mechanism (Fernandez et al., 2001). The IR inactivation in both skeletal muscle and adipose tissue did not cause diabetes, but peripheral insulin resistance accompanied by mild β-cell dysfunction (Lauro et al., 1998).

*Loss of IR in of β-cell:* when IR gene was disrupted specifically in the β-cells of the pancreas, insulin secretion was lost in response to glucose. In addition, it progressed into glucose intolerance; this means that insulin activates its own secretion through IR on β-cells (Kulkarni et al., 1999). The loss of IR on β-cells causes alteration in insulin signaling in β-cells which contributes to the development of impaired insulin secretion and T2DM (Kulkarni et al., 1999).

*Insulin receptor in the brain:* loss of IR in brain causes mild insulin resistance and diet-induced obesity, however, the brain develops normally (Bruning et al., 2000).

*Loss of Insulin Receptor Substrates (IRSs):* Loss of IRS-1 in mice caused glucose intolerance and insulin resistance (Abe et al., 1998; Previs et al., 2000).

**Role of Phosphatases**

Another possible mechanism to induce insulin resistance is the role of the protein tyrosine phosphatases (PTPs) (Goldstein et al., 1998a; Tonks and Neel, 2001; Zhang, 2001). These proteins decrease insulin action by terminating phosphorylation of IR and IRS (Calera et al., 2000; Cheng et al., 2002; Goldstein et al., 2000). PTP-1B is one of
these PTPs that is present in insulin responsive tissues (liver, muscle, adipose tissue and brain) (Cheng et al., 2002; Goldstein et al., 1998a; Goldstein et al., 1998b). It is overexpressed in insulin resistant animal models (Ahmad and Goldstein, 1995; Taghibiglou et al., 2002) and in obesity (Ahmad et al., 1997a; Ahmad et al., 1997b; Ahmad and Goldstein, 1995; Goldstein et al., 1998b; Taghibiglou et al., 2002; Wu et al., 2001).

**Visceral Obesity and Insulin Action**

*Epidemiology of obesity*

The WHO has recognized that obesity is on the rise and epidemic world-wide. The estimated numbers are 1 billion people are considered to be overweight, and 300 billion to be obese (Smyth and Heron, 2006). The major causes of this increase in the number of obese and overweight people include their sedentary life style, increased high-fat intake and diets rich of energy, and the modern life style (Smyth and Heron, 2006). The National Health and Nutrition Examination Survey has conducted a study in the U.S. in 1999-2000 and concluded that around 64.5% of the adult Americans are either overweight or obese with a body mass index (BMI) of 25 or more (Smyth and Heron, 2006).

The best marker that is widely used to describe obesity is the BMI which is calculated as the body weight in kilograms divided by the square height in meters. According to this definition, a BMI of 25-30 kg/m² is overweight, 30-40 kg/m² is
considered obese subject, 40-50 kg/m² is morbid obesity, and a BMI over 50 kg/m² is considered as extreme obesity (Mlinar et al., 2007).

There is a world-wide increase in obesity which is strongly associated with insulin resistance, T2DM (Plum et al., 2005), increased risk of cardiovascular diseases, liver diseases, immune and reproductive dysfunctions, orthopedic disorders and some kinds of cancer (Vague, 1996). In this respect, recent research has focused on the molecular mechanisms that link adipose tissue to insulin sensitivity in the whole body, β-cell function, and overall glucose metabolism. It is known that insulin receptor plays an important role in adipocyte growth and differentiation (Accili et al., 1996). This was observed in newborn mice with insulin receptor deficiency which showed lower WAT mass (Accili et al., 1996; Joshi et al., 1996). Adipose tissue-specific knockout of insulin receptor in mice (FIRKO) showed a lower fat mass and loss of the correlation between plasma leptin and body weight, and protection against both obesity and obesity-related glucose intolerance (Bluher et al., 2002).

**Development of Obesity**

Obesity is a heterogeneous polygenic disorder with unclear exact cause. However, obesity develops as a result of interaction between several genetic and environmental factors. It mostly occurs when there is chronic imbalance between caloric intake and energy expenditure in the body (Porte, 2003). It is estimated that about 40-80% of obesity cases have underlying genetic basis (Bell et al., 2005) as suggested by too many studies that confirmed the correlation between obesity and several putative gene loci (Perusse et
al., 2005). Food intake, lack of physical activity and smoking are examples of environmental factors that are implicated in the development of obesity (Cummings and Schwartz, 2003).

Fat distribution is considered more important than total fat mass because of differences in tissue-specific responses to hormones (Arner, 1997; Bonora et al., 1992; Pouliot et al., 1992). Based on differences in their response to different hormones and their anatomical location, fat tissue can be classified into two types: android or male-type, and gynoid or female-type fat. In the earlier, fat is mainly present in the upper part of the body in the abdominal region, while in the latter fat is mostly in the gluteo-femoral region (Vague, 1996; Vague et al., 1974). Abdominal fat is composed of abdominal subcutaneous and intra-abdominal fat which is composed of visceral (intraperitoneal) and retroperitoneal fat (Abate et al., 1994).

**Visceral Versus Subcutaneous Obesity**

Visceral adipose tissue is different from subcutaneous and has more negative effect on insulin action and some other metabolic abnormalities. First, certain receptors are widely expressed in visceral adipose more than subcutaneous tissue. Angiotensin II receptors type-1 (AT1), β1-, β2-, β3-adrenergic receptors, glucocorticoids, and androgen receptors are all examples of receptors that are expressed in visceral adipose tissue more than it subcutaneous (Arner, 1995; DeFronzo, 2004; Vohl et al., 2004). Second, visceral adipose tissue secretes more IL-1β and plasminogen activator inhibitor-1 (PAI-1) which are involved in insulin resistance, however, leptin and adiponectin are secreted more from
subcutaneous adipose tissue (Kershaw and Flier, 2004). Third, increased insulin resistance in visceral adipose tissue compared to subcutaneous is also due to higher levels of antilipolytic insulin receptors, $\alpha$-2A adrenergic receptors, and estrogen receptors in subcutaneous tissue (Arner, 1995; Vohl et al., 2004). Fourth, adipose tissue-derived factors are directly released to liver through the portal circulation, where they can contribute to insulin resistance by increasing gluconeogenesis, lipogenesis and decreased glucose uptake, which all contribute to the overall insulin resistance. Although increased adipose tissue mass plays the most important factor in the development of insulin resistance, some studies demonstrated that certain amount of adipose tissue should be present for the normal insulin sensitivity particularly the adipose tissue-derived hormones adiponectin and leptin (Shimomura et al., 1998; Yamauchi et al., 2001).

**Fat Storage and Mobilization**

In order to understand the effect of fat metabolism on insulin action, we should understand how FFA are metabolized and activate metabolic pathways. In addition, we need to study what receptors can regulate the action of FFA.

The causal-effect relationship between T2DM, HF-intake and obesity is not fully elucidated. But it is well known that visceral adiposity is strongly related to T2DM (Pascot et al., 2001). Absorption of fat from the diet occurs with no control, which means whatever is present in the diet, will be absorbed into the blood circulation. However, metabolism of fat, as other metabolic processes, is regulated by genetic, hormonal and nutritional factors (Lewis et al., 2002). The FFA level in circulation is regulated by the
amount of fat transported into and that is mobilized from adipose tissue. Uptake of FFA occurs via esterification in adipose tissue and liver and oxidation in muscle, liver and other organs. Adipocytes utilize insulin to increase glucose uptake, triglyceride synthesis and decreased FFA release to circulation via inhibition of lipolysis. High plasma FFA result in reduced glucose oxidation, as there is competition for oxidation. Since adipose tissue is very sensitive to changes in insulin, it takes little insulin to proliferate more and hypertrophies up to a limit beyond which it becomes hard to store more FFA. This leads to release of FFA in the bloodstream and targets the insulin sensitive tissues. As a result, insulin resistance develops, a usual abnormality seen in obesity and before the development of T2DM and afterward (Mantzoros and Flier, 1995). Furthermore, FFA release occurs from the triacylglycerol stored in lipoproteins, and from lipolysis of the adipose tissue depots induced by the Hormone Sensitive Lipase (HSL). This enzyme is reduced in hyperinsulinemia (Lewis et al., 2002) and there is also defective esterification and re-esterification of fatty acids in adipose tissue in insulin resistance and T2DM. The rate of FFA release depends on the tissue where adipocytes are, with a greater rate from visceral adipose tissue (Lewis et al., 2002). The increased rate of release from this tissue is important because it releases FFA directly into the portal circulation. This leads to increased flux of FFA to liver, with subsequent elevation in hepatic triglyceride synthesis and hepatic glucose output. These two are directly related to insulin resistance and T2DM. Due to the increased insulin resistance, pancreas will respond by secreting more insulin to overcome insulin resistance in these tissues.
The relationship between glucose and fatty acid metabolism was introduced by Randle (Randle, 1998; Randle et al., 1963). When FFA levels increase, their uptake, oxidation and storage is increased. This will inhibit glucose oxidation to increase its storage when glycogen reserves are low. On the other hand, malonyl-CoA works to downregulate mitochondrial fatty acid oxidation by inhibiting CPTI; this will maintain glucose oxidation up. ACC-derived malonyl-CoA also serves as FAS substrate for fatty acid synthesis. Decreased ACC activity and malonyl-CoA levels in diabetes, obesity and insulin resistance is associated with increased fatty acid oxidation (McGarry et al., 1978; Ruderman et al., 1998). Similarly, insulin-resistance states are associated with desensitization of CPTI to malonyl-CoA (Zammit, 1996). Thus it is possible that by phosphorylating CEACAM1, insulin acutely decreases FAS activity to preserve malonyl-CoA levels to lower fatty acid oxidation and maintain that of glucose.

*Adipose Tissue as an Endocrine Organ*

Visceral adipose tissue secretes FFA, hormones and inflammatory cytokines that play a key role in the pathogenesis of insulin resistance in obesity and T2DM through their effect on insulin action in insulin target tissues as well as vasculature (Arner, 2001; Krebs and Roden, 2005; Ruan and Lodish, 2004). The liver is the primary target which is affected by these visceral adipose tissue-derived effectors including FFA, TNF-α (Hotamisligil et al., 1993), IL-6, IL-1β, iNOS (Coppock, 2001; Frayn et al., 2003), resistin (Steppan et al., 2001), and decreased adiponectin (Berg et al., 2001). In light of
these effects on liver it becomes imperative to further study the mechanisms by which these effectors can modulate insulin action in liver.

It has become evident that obesity involves macrophage infiltration into the adipose tissue (Weisberg et al., 2003; Xu et al., 2003). Several adipocyte-derived factors have been shown to contribute to insulin action and resistance. Adipose tissue-derived FFA have been shown to contribute to insulin resistance in liver and muscle in obesity (Bergman and Ader, 2000; Boden, 2006). In addition to leptin, adipose tissue secretes a large number of proteins that affect insulin action (Kershaw and Flier, 2004; Rajala and Scherer, 2003). Leptin, adiponectin, and visfatin (Pre-B cell colony enhancing factor: PBEF) are adipose tissue derived proteins that improve insulin sensitivity (Fukuhara et al., 2005; Kershaw and Flier, 2004; Rajala and Scherer, 2003). Adipsin/ASP (Acylation Stimulatory Protein), resistin, TNF-α, IL-6, MCP-1 (Macrophage and monocyte chemoattractant protein-1), PAI-1 (Plasminogen activator inhibitor-1), angiotensin and others have been shown to contribute to insulin resistance (Fukuhara et al., 2005; Kershaw and Flier, 2004; Rajala and Scherer, 2003). Circulating levels of these proteins were elevated in human studies of T2DM (Lazar, 2005).

**Resistin**

A polypeptide hormone expressed in adipocytes in mice (Steppan et al., 2001) and macrophages in humans (Patel et al., 2003), usually elevated in insulin resistance states.
**Role of Adipokines in Insulin Action**

Visceral obesity and insulin resistance are now considered as chronic subacute inflammatory states that are associated with elevated levels of fat-derived adipokines (Kershaw and Flier, 2004; Lazar, 2005; Sjoholm and Nystrom, 2005; Tataranni and Ortega, 2005) such as TNF-α, IL-6, IL-1β, iNOS, and others (Coppock, 2001; Frayn et al., 2003). These adipokines (TNF-α, IL-1β and IL-6) are known to impair insulin action and signaling (Coppock, 2001; Kershaw and Flier, 2004; Marette, 2002). However, when mice were fed with HFD, insulin resistance did develop several months before the involvement of these adipokines (Xu et al., 2003).

Macrophage infiltration to the adipose tissue in obesity is participating in the production of these cytokines to play a role in development of insulin resistance (Weisberg et al., 2003; Xu et al., 2003). Therefore, macrophage markers (e.g., CD 68) increased significantly in HFD-fed mice (Xu et al., 2003). Some cytokines such as TNF-α, IL-6, and others are secreted from macrophages and adipocytes. They can directly act on liver to produce acute phase proteins or indirectly through activation of other inflammatory cells. For example, these cytokines can secrete SOCS-3 (Suppressor of cytokine signaling-3), which is an intracellular protein that impairs signaling of both insulin and leptin (Shi et al., 2004).

The activation of the inflammatory pathway NF-κB is associated with macrophages, liver, and vascular cells amid others (Weisberg et al., 2003; Xu et al., 2003) in visceral obesity.
That insulin resistance is caused by increase in the pro-inflammatory state that is associated with visceral obesity has been demonstrated in several epidemiological and longitudinal studies (Calle and Kaaks, 2004; Chen et al., 2004) in humans as well as in several transgenic and knockout mouse models, in particular those with hyperactivated IKK-β and NF-κB pathways in addition to TNF-α, IL-6 and iNOS signaling pathways (Cai et al., 2005; Hirosumi et al., 2002; Kim et al., 2004; Perreault and Marette, 2001; Uysal et al., 1997; Yuan et al., 2001).

**Tumor Necrosis Factor-α (TNF-α) and Insulin Action**

The tumor necrosis factor-α (TNF-α) is upregulated in obesity (Kern et al., 2001; Moller, 2000) and induces “chronic low-grade inflammation” (Sell et al., 2006a) in adipose tissue of obese patients (Sjoholm and Nystrom, 2006). Some *in vitro* studies have shown that TNF-α induces insulin resistance in liver cells and adipose tissue (Rotter et al., 2003; Senn et al., 2002). It is believed that TNF-α induces this negative effect on insulin action by decreasing tyrosine phosphorylation of IRS proteins via increased serine phosphorylation (Hotamisligil et al., 1996; Le Marchand-Brustel et al., 2003; Sykiotis and Papavassiliou, 2001) which ultimately leads to attenuation of insulin action, and by decreasing adiponectin secretion (Ruan and Lodish, 2004).

**Interleukin-6 (IL-6) and Insulin Action**

Interleukin-6 is upregulated in obesity and induces inflammation in adipose tissue of obese subjects (Sjoholm and Nystrom, 2006). The effect of IL-6 on insulin signaling is
skeletal muscle is still under debate because of discrepancy in studies in which some showed enhanced insulin sensitivity while some others showed increased insulin resistance after IL-6 treatment (Rieusset et al., 2004; Weigert et al., 2005).

**Leptin and Adiponectin**

Leptin and adiponectin are both adipokines that are mainly secreted from adipocytes (Trujillo and Scherer, 2005; Zhang et al., 2005). Leptin was discovered in 1994 as adipocyte-secreted protein, when deficient it causes morbid obesity and T2DM (Zhang et al., 1994). It is a 16 KDa protein produced by WAT and regulates food intake. Leptin mediates the innate adaptive neuroendocrine response to starvation in physiological conditions. However, when adipose tissue is depleted its levels decrease which stimulates the desire to seek food and suppress the thyroid, growth hormone, adrenal and reproductive endocrine axes (Ahima and Osei, 2004).

Leptin is a hormone that controls body weight (Zhang et al., 2005) by affecting food intake. The deficiency of leptin, as in the \textit{ob/ob} mice is associated with obesity, insulin resistance, and diabetes (Kahn and Flier, 2000). When these \textit{ob/ob} mice and rats where treated with leptin, insulin sensitivity has improved and resulted in reversal of obesity and diabetes (Chinookoswong et al., 1999; Muzzin et al., 1996). However, some studies were unable to show this effect of leptin in improving insulin sensitivity (Widdowson et al., 1998; Zierath et al., 2000), hence the difficulty in finding a clear relationship between leptin and insulin resistance. In addition, when lipodystrophic humans and mice were treated with leptin their insulin resistance and diabetes were
corrected (Javor et al., 2005; Shimomura et al., 1999). However, in vitro leptin showed impaired insulin action in liver, adipose, and muscle tissue culture cells (Cohen et al., 1996; Muller et al., 1997; Sweeney et al., 2001). Therefore, it is still unclear whether the effects of leptin are due to hyperleptinemia or due to selective brain leptin resistance (Arch, 2005).

Adiponectin is secreted from adipocytes; however, it is downregulated in obesity and is considered the only adipokine that enhances insulin sensitivity (Lihn et al., 2005). Therefore, in obesity where plasma adipokine levels are reduced, this can be used as a marker of insulin resistance and possibility of developing T2DM (Tschritter et al., 2003). Some in vitro studies have shown that adiponectin can regulate adipocyte secretions including adipokines which prevents insulin resistance in muscle cells co-cultured with adipocytes (Dietze-Schroeder et al., 2005). Therefore, adiponectin is envisioned as a regulator of insulin resistance inducing adipokines from adipocytes and makes a balance with these adipokines (Nawrocki and Scherer, 2004).

**Other Cytokines**

Other cytokines affect insulin action, however, the mechanism is not as clear (Coppack, 2001; Kim et al., 2004; Marette, 2003). For example, monocytes and endothelial cells secrete Monocyte chemotactic protein-1 (MCP-1) which is linked to obesity (Sartipy and Loskutoff, 2003; Takahashi et al., 2003) and atherosclerosis have been shown to cause insulin resistance in adipocytes (Sartipy and Loskutoff, 2003) and muscle cells (Sell et al., 2006b). In addition, recent studies on primary human adipocyte
have found some new adipokines that are secreted from adipocytes and are implicated in the development of insulin resistance, such as the tissue inhibitor of metalloproteinases-1 (TIMP-1) and retinol-binding protein-4 (RBP-4) (Sartipy and Loskutoff, 2003; Yang et al., 2005). Muscle tissue can release secretory products known as “myokines” that modulate the ratio of lean to fat mass and consequently insulin resistance through their muscle-to-fat signaling (Sell et al., 2006a). Examples are IL-6 secretion in skeletal muscle which has been shown to have positive effect on insulin sensitivity in muscle during exercise (Weigert et al., 2005) and IL-15 that alters muscle protein metabolism (Carbo et al., 2001). It reduces adipose tissue mass by favoring FFA oxidation (Almendro et al., 2006) and increases the secretion of adiponectin in 3T3 adipocytes (Quinn et al., 2005).

**The Role of Visceral Adipose Tissue-derived FFA on Insulin Action**

Most of T2DM patients are obese and show increases plasma FFA levels (Boden, 2006). This chronic elevation of plasma FFA is considered as an independent risk factor for development of glucose intolerance and its progression to T2DM (Baldeweg et al., 2000; Charles et al., 1997; Knowler et al., 1990). Consistently, it is likely that reduction of plasma FFA levels ameliorates insulin resistance and improves the lipid profile in diabetic patients. Insulin normally causes suppression of FFA release from adipose tissue. In case of insulin resistance this suppression is impaired and leads to increase in FFA flow to the liver. This in turn causes overproduction of VLDL and elevation of plasma triglycerides (Carmena, 2005).
Several *in vivo* studies (Groop et al., 1989; Shulman, 2004) support the notion that increased FFA are correlated with insulin resistance (Groop et al., 1991). High plasma FFA levels resulted from HF-intake, reduced cellular uptake, or increased release can induce insulin resistance (Lewis et al., 2002). This is seen in cases where storage and/or mobilization of fat from storage sites are abnormal at very early stages of insulin resistance. Fat metabolism continues to be impaired in obesity, insulin resistance and T2DM. In response to this, β-cells of the pancreas start to secrete more insulin to compensate for the resistance, which leads to hyperinsulinemia. Hyperinsulinemia may also result from reduced insulin clearance in the liver to maintain insulin sensitivity (Valera Mora et al., 2003).

Free fatty acids are mobilized from visceral adipose tissue more than subcutaneous tissue due to the lower sensitivity to the antilipolytic effect of insulin (Ostman et al., 1979). Their mobilization could take place even before insulin resistance develops in these tissues (Lewis et al., 2002). Once they are released visceral adipose tissue to the liver via the portal vein during fasting or excessive fat intake, FFA can be preferentially removed by PPARα oxidation (Ferre, 2004) or oxidized to a lower extent in muscle, heart, and other tissues (Lewis et al., 2002).

In liver, mobilized FFA can contribute to insulin resistance (Bays et al., 2004; Bergman and Ader, 2000; Kabir et al., 2005; Rebrin et al., 1995) via different mechanisms: promoting gluconeogenesis (Massillon et al., 1997; Williamson et al., 1966), increasing FFA oxidation and lipogenesis (Oakes et al., 1997; Sidossis et al., 1998), decreasing glycogenolysis that is normally inhibited by insulin (Boden et al., 2002), and
by reducing insulin clearance (Mittelman et al., 2000). In muscle, FFA uptake and oxidation competes with that of glucose and results in hyperglycemia and insulin resistance (Randle et al., 1963).

Several molecular mechanisms have been suggested by various studies to explain how FFA release from adipose tissue can induce insulin resistance and impair action (Lewis et al., 2002; Smith, 2003). These mechanisms reveal that FFA and their metabolites work either directly or indirectly, by activating inflammatory signals, to impair insulin signaling pathways, contribute to liver steatohepatitis and vascular disease and hypertension (Boden, 2006). For example, increased plasma FFA levels lead to reduction in IRS-1 protein (Smith, 2003), decreased activation of PI3-Kinase-PKB/Akt pathways, decreased activity of certain atypical PKC isoforms which work to switch on GLUT-4 translocation/glucose transport responses, decrease in fat cell GLUT-4 content, decrease in insulin receptor kinase activity and phosphorylation of the insulin receptor (Smith, 2003). High-fat intake increases plasma FFA levels, which leads to increase triglycerides in liver and muscle. This effect on liver leads to hyperglycemia due to increased glucose output (Schmitz-Peiffer et al., 1997). While in muscle, it leads to higher levels of DAG which activates PKCε and θ that in turn results in decreased phosphorylation of IRS-1 and in glycogen synthase activity. The latter leads to reduction in glucose uptake which ultimately causes hyperglycemia. Acute lipid infusion increases FFA levels and causes mild inflammation as indicated by activation of NF-κB pathway (IKKβ and NF-κB), and higher secretion of inflammatory cytokines (TNF-α and IL-1β) and MCP-1. Obese and diabetic humans showed impaired PI3-kinase activity as well as
activation of PKCε and λ (Kim et al., 2003), which is consistent with the hypothesized mechanism of insulin resistance. Other mechanisms of insulin resistance induced by elevation of plasma FFA levels includes the activation of the JNK pathway and hypersecretion of TNF-α (Nguyen et al., 2005). In addition, excessive saturated, but not mono-unsaturated fat, leads to accumulation of ceramide which in turn inhibits the Akt in muscle. Inhibition of Akt subsequently interferes with glucose uptake in response to insulin in muscle and which induces insulin resistance (Chavez et al., 2003).

**Visceral Adiposity and Insulin Clearance**

As discussed earlier, insulin resistance is associated with visceral obesity which induces more insulin secretion to overcome the resistance (Polonsky et al., 1988; Valera Mora et al., 2003). On the other hand impairment of insulin clearance has been shown to be a major cause of hyperinsulinemia in obesity (Bonora et al., 1983; Escobar et al., 1999; Giugliano et al., 1993; Hansen et al., 1993; Peiris et al., 1986; Polonsky et al., 1988; Rossell et al., 1983) and hypertension (Giugliano et al., 1993). This was evident from several studies conducted in humans and animals, in which 10% loss of body weight was associated with enhanced insulin clearance and sensitivity compared to insulin secretion which needs more significant weight loss and recovery of insulin resistance (Jones et al., 2000). *In vivo* studies in human subjects have suggested that simple obesity in causes hyperinsulinemia due to increased insulin secretion and due to reduced hepatic insulin clearance, and that the effect of reduced hepatic insulin clearance becomes more significant when the severity of glucose intolerance becomes higher (Bonora et al., 1983).
Most of in vivo studies have suggested that elevation of plasma FFA levels in the portal circulation is the main factor in causing impairment of insulin clearance in obesity (Balent et al., 2002; Carpentier et al., 2000; Carpentier et al., 1999; Svedberg et al., 1991; Wiesenthal et al., 1999). These suggestions were supported by further studies in dogs where FFA were acutely infused into the portal circulation which interestingly showed more deterioration of insulin clearance more than when they were peripherally infused (Yoshii et al., 2006). The imperative question is how FFA can regulate insulin clearance? Indeed, the exact answer to this question is not yet clear (Lewis et al., 2002). However, some mechanisms suggested a decrease in insulin binding to its receptor (Hennes et al., 1990; Svedberg et al., 1990), direct association of FFA to insulin degrading enzymes (IDEs) that leads to their inhibition (Estrera et al., 2001), activation of PKCδ which is believed to affect insulin internalization (Chen et al., 2006), and ligand-induced receptor downregulation (Hennes et al., 1990; Svedberg et al., 1990) as a result of chronic hyperinsulinemia and increasing insulin delivery to the portal circulation (Carpentier et al., 2000; Carpentier et al., 1999). Our laboratory has shown that CEACAM1 promotes hepatic insulin clearance and that it has reduced activity associated with visceral obesity and insulin resistance (Poy et al., 2002b).

The FFA have variable degrees of saturation which plays a crucial role in their effect on insulin action, they could be saturated fatty acids (SFA), mono-unsaturated (MUFA), saturated fatty acids (SFA) such as palmitic acid are showing the most negative effect on insulin action (Dobbins et al., 2002; Vessby et al., 1994; Xiao et al., 2006) compared to omega-3 (n-3) polyunsaturated like linolenic acid, DHA and EPA which all
have a protective effect against insulin resistance induced by SFA (Borkman et al., 1993; Storlien et al., 1987; Vessby et al., 2001). The effect of MUFA such as oleic acid is generally intermediate and less than that of n-6 PUFA, such as linoleic acid. Although the variable effects of these FFA are not so clear, they are, however, believed to work on several signaling pathways. For example, PUFA, but not SFA or MUFA can regulate hepatic lipogenic enzymes by inhibiting the activity of the transcription factor SREBP-1 (Xu et al., 1999). In addition, PUFA have more capacity to induce and activate the transcription factor PPARα, which regulates fatty acid oxidation in liver and β-cells in the pancreas. Compared to PUFA, SFA do not activate PPARα, while MUFA have intermediate activation effect (Kliewer et al., 2001). Furthermore, PUFA and MUFA members have variable effects on PPARα due to their degree of binding and activation (Krey et al., 1997). In vivo studies in humans (Carpentier et al., 2000) and dogs (Wiesenthal et al., 1999) have shown that intravenous infusion of lipids, which primarily consists of linoleic acid (n-6 PUFA), decreased insulin clearance. Interestingly, this decrease was observed both after acute (5-7 h) and prolonged (24 h) infusion, and that this decrease happened to prevent insulin deficiency that might happen as a result of reduced glucose-stimulated insulin secretion. Linoleic acid-rich PUFA ingestion for 24 h increases its plasma level and induces impairment of insulin clearance in obese non-diabetic subjects much more than MUFA, which increased plasma oleic acid, and SFA, which increased plasma stearic and palmitic acids (Xiao et al., 2006). This reduction in hepatic insulin clearance occurs to compensate for the reduction of glucose-stimulated insulin secretion that might happen in obese subjects at risk.
Figure 6. **Insulin Resistance is Secondary to Impairment of Insulin Clearance Due to Reduced Hepatic CEACAM1 Levels**

Proposed mechanism of insulin resistance caused by reduced hepatic CEACAM1 levels. HF-diet leads to visceral adiposity which increases FFA release to portal circulation. As a result CEACAM1 levels will be reduced leading to impairment of insulin clearance and, hyperinsulinemia which in turn leads to insulin resistance.
**Peroxisome proliferators activated receptors**

Peroxisome Proliferator Receptors (PPARs) play an important role in lipid metabolism. These are members of the nuclear hormone receptor transcription factors. There are three isoforms of this family; PPARα, β/δ and γ. The PPARα is expressed in liver, heart, kidney, skeletal muscle, brown fat, monocyctic, vascular endothelial and vascular smooth muscle cells (Berger and Moller, 2002). They control gene expression of their target genes by binding to specific sites in PPAR Response Elements (PPREs) in the promoter region of their target genes to activate their transcription. The PPRE of the PPAR gene is most commonly consisting of the sequence (A/G)GGTCA directly repeated with a one nucleotide spacer (DR-1 elements) (Everett et al., 2000). This PPRE is present in all promoters of the target genes of the enzymes involved in peroxisomal fatty acid β-oxidation (Latruffe et al., 2001). Activation of this leads to induction of battery of enzymes involved in lipid metabolism and regulation. They can be activated by natural ligands (fatty acids and eicosanoids) as well as some synthetic compounds. Upon ligand binding, they heterodimerize with retinoid x receptors (RXR) and a conformational change occurs in the PPAR. This creates a binding cleft, with recruitment of co-activators. Then they bind to the PPRE in the target gene to enhance gene transcription (Berger and Moller, 2002). The PPARα is involved in cellular uptake and β-oxidation of fatty acids. Many genes are upregulated via PPARα activation: FATP and FAT, LCFA acetyl-CoA Synthase, ACO, enoyl-CoA Hydratase/dehydrogenase, Ketoacyl-CoA thiolase, CPT I and many others important in lipid metabolism (Berger and Moller, 2002). This isoform is activated by SFAs, MUFAs and PUFAs, as well as the
synthetic antihyperlipidemic agents (fibrates). Anath Shalev and co-workers (Shalev et al., 1996) have shown that PPARα is a phosphoprotein that undergoes phosphorylation in response to insulin, with increase in transcriptional activity as well. This supports the idea that there is a cross-talk between insulin signaling and PPARα activation. This link might serve as a molecular basis for the relation of insulin-resistance and altered lipid metabolism (Shalev et al., 1996) PPARβ/δ on the other hand is ubiquitously distributed, and has no clear functions. PPARγ is mainly expressed in adipose tissue and is responsible for adipocyte differentiation. It also regulates numerous numbers of genes involved in energy homeostasis and genes that affect insulin action (Berger and Moller, 2002). The TNF-α, C-CBL, IRS-2 and many others are among the genes that are regulated by PPARγ. They are activated by fatty acids, mainly the PUFAs, as well as the oral antidiabetic drugs thiazolidinediones (TZDs), which enhance insulin activity as insulin sensitizers (Berger and Moller, 2002).

**Does Obesity Cause Diabetes?**

Adipose tissue is widely recognized as an endocrine organ that secretes hormones, FFA, cytokines and other effectors. The secretion of these hormones is suggested to play a role in the communication between the brain and peripheral tissues to regulate appetite and metabolism (Kershaw and Flier, 2004). The characteristics of the adipose tissue in terms of the location (visceral versus subcutaneous) (Das et al., 2004), size of adipocyte (Weyer et al., 2001b), and adipocyte metabolism of glucose (Abel et al., 2001) and glucocorticoids (Masuzaki et al., 2001) appear to modulate the functions of the adipose
tissue-derived effectors. Stimulation of the innate immune response by bacterial endotoxin during sepsis (Agwunobi et al., 2000) has led to insulin resistance. This is an example that shows the relationship between inflammation and the development of diabetes as inflammation interacts with insulin signaling. This observation was supported by the ability of aspirin to improve insulin resistance, partly by preventing the negative effects of FFA and cytokines on insulin signaling in inflammatory states (Yuan et al., 2001). Intracellular stress has been shown to induce insulin resistance which is exacerbated by obesity, because of the adipose tissue-derived effectors that lead to inflammation (Ozcan et al., 2004). In addition, chronic elevation in these inflammatory adipokines negatively affect β-cells in the pancreas which ultimately causes insufficient insulin secretion to overcome insulin resistance (Rhodes, 2005). Insulin resistance, which is the hallmark of T2DM, and the subsequent failure of insulin secretion are evident of T2DM.

Obesity and T2DM are strongly associated with an estimation of (≥ 80%-90%) of T2DM in the US are overweight or obese (Mokdad et al., 2000) Whether lean or obese, there is a chronic elevation in plasma FFA levels that do not fall back into normal levels after mixed meal or an oral glucose load (Reaven et al., 1988). The FFA are released from their TG storage sites in adipose tissue under the control of hormone-sensitive lipase (HSL) which is inhibited by insulin (Bays et al., 2004; Groop et al., 1989). However, this inhibition is impaired in case of T2DM and in obese subjects.


Materials and Methods

Quantitative RT-PCR for Cecam1 Analysis in Rat Models

Liver and kidney were collected and snap frozen from male rats (Zucker males $fa/fa$ and $fa/+$/ of 7-8 wk old, 8 and 16 wk Koletsky male rats (lean and obese counterparts) and 6 and 12 wk old male ZDF (lean and obese).

From the frozen tissues, 350 mg were homogenized using Teflon homogenizer. The total RNA was extracted by TRIZol (GIBCO BRL). MicoPoly (A) pure Kit (Ambion) was used for purification of total mRNA. First strand cDNA was synthesized using Super script II and oligo dT. To characterize the expression pattern of the different isoforms of CEACAM1 in the liver and kidney we used the Real-Time PCR quantification by applied BioSystem. Three pairs of primers were designed, for short isoform (data not shown), long isoform (CEACAM1, F: 5’CAGCGCTGGCATACTTCCT3’, R: 5’CACTTCCCCCGCCAGTCTT3’), and $\beta$-actin (F: 5’ATCAAGATCATTGCTCCTCTCTGA3’, R: 5’GAGCCACCAATCCACACAGAG3’) as endogenous reference. At least of one primer of each primer pair is located in junction of two exons to avoid amplification of genomic DNA, the concentration of primers were optimized to 50 nM. The reaction was performed in total volume 50 µl with a reaction condition of DNA polymerase activation 95° C 10 min, denaturation 95°C 15 sec and annealing/extension 60°C for 1 min for 40 cycles. To confirm the absence of non-specific amplification, the product of non-template control was analyzed by agarose gel electrophoresis. For absolute quantification of unknown samples, standard curves were prepared for both the target and the endogenous reference. The amount of the target
molecules were determined using standard curves. These values were normalized with the endogenous references (β-actin).

Animal Maintenance and High-fat Feeding

Animal husbandry: Animals were kept in a 12-h dark/light cycle and fed either a standard chow ad libitum or a high-fat (HF) diet which provides 45% of calories from fat and 17% from sucrose (Research Diets). The fat composition of the diet was 36.3% saturated; 45.3% MUFA and 18.5% PUFA. For CEACAM1 and insulin action assessment, mice were placed on either diet for 9-30 d with free access to food. All procedures were approved by the Institutional Animal Care and Utilization Committee at the College of Medicine at the University of Toledo. All mice were from the BL6 background and fasted unless otherwise mentioned.

Phenotypic and Biochemical Characterization of Animals

Following an overnight fast (with food being removed at 5:00 p.m. on the day prior to the experiment), mice were anesthetized with IP sodium pentobarbital (40 mg/kg body weight) between 11:00 a.m. and 12:00 p.m. Whole venous blood was drawn from the retro-orbital sinuses to measure random or fasting glucose levels using a glucometer (Accu-Check, Roche Applied Science), plasma insulin, C-peptide and leptin levels by radioimmunoassays (Linco Research), plasma FFA using the NEFA C kit (Wako) and plasma triglycerides (Pointe Scientific). Visceral adipose tissues were weighed, and visceral adiposity was expressed as percentage of total body weight.
Intra-peritoneal Glucose Tolerance Tests (IPGTT)

Whole-blood glucose was determined 0-120 min after glucose injection (2 mg/kg body weight) of age-matched 4-mo-old overnight fasted male mice, and as described previously (Poy et al., 2002b).

Liver and Muscle Triglyceride Content

Liver and muscle triglyceride content was determined in homogenized tissues by extracting lipid with CHCl₃:CH₃OH (2:1, v/v), drying under N₂, resuspending in ethanol, and measuring triglyceride levels as the difference between free and total glycerol, as described previously (Dai et al., 2004).

Western Blotting

To determine the protein content, 75 μg lysates from frozen tissues were analyzed directly by SDS-PAGE followed by sequential immunoblotting with their antibodies, and then with either monoclonal α-actin or monoclonal α-tubulin (Sigma-Aldrich) to normalize for the amount of proteins loaded on the gel. Autoradiograms were scanned for band density measurement versus that of actin or tubulin and expressed as arbitrary units. CEACAM1 in liver or cellular lysates were analyzed using a polyclonal antibody against mouse CEACAM1 (α-mCC1). The FAS content was analyzed using a polyclonal antibody against FAS (α-FAS). Antibodies for PPARα, CD36, and FATP-1 antibodies were obtained from Santa Cruz. The levels of phospho-Akt (p-Akt, Ser⁴⁷³) were assessed via western blotting, after pre-activation with insulin or buffer alone in the
presence of cold ATP, using an antibody against p-Akt then re-probed with α-Akt antibody to normalize per amount of p-Akt (both antibodies were purchased from (Cell Signaling Technology). PTP-1B levels were determined using an antibody from (Upstate).

**Insulin Receptor Phosphorylation**

We dissected tissues from anesthetized animals and froze them immediately in liquid nitrogen before being stored at -80°C freezer. Then the tissues (liver, soleus muscle or WAT) from different groups were homogenized in ice-cold lysis buffer (1% Triton-X-100 in the presence of Phosphatase and protease inhibitors) (Najjar et al., 1993). For phosphorylation *in vitro*, lysates were treated with 100 nM Insulin (Sigma-Aldrich) or buffer alone for 40 min prior to phosphorylation in the presence of cold ATP. Proteins were immunoprecipitated with anti-insulin receptor β-subunit antibody (α-IRβ) (Santa Cruz Biotechnology Inc.). Following immunoprecipitation, proteins were analyzed on SDS-PAGE and transferred into nitrocellulose membranes prior to detection of phosphorylation by a monoclonal anti-phosphotyrosine antibody (α-pTyr) (Upstate). The blots were reprobed with α-IRβ to account for the amount of insulin receptor in immunopellets.

**Assessing Transcript Levels in Adipose Tissue**

*Preparation of the cDNA:* 30 mg of adipose tissue from mice were homogenized using a mortar on liquid nitrogen to a fine powder. The mRNA was isolated using a μMACS One-step cDNA Kit from MACS, Molecular Biology reagents (Miltenyi Biotec
Inc., CA). After lysing the samples according to the manufacturer protocol, mRNA was isolated after addition of Oligo (dT) MicroBeads. Then proceed to synthesize the cDNA using the kit. The PCR analysis of transcript levels was performed in a temperature fluorescence temperature cycler (LightCycler; Roche). Briefly, amplification was performed in a 10-µl final volume containing 50 mM Tris (pH 8.3), 3 mM MgCl2, 4.5 mg of bovine serum albumin, 200 mM deoxynucleotide triphosphates, a 1:20,000 dilution of SYBR Green I (Molecular Probes), 1 mM each primer, and 1 U of platinum Taq polymerase (Invitrogen). Amplification was performed for 40 cycles, with each run consisting of an initial melting at 95°C for 2 min followed by melting, annealing, extension, and acquiring temperatures specific to each primer set. Serial dilutions of a representative template cDNA were amplified using each primer set (CD68; F: 5’-CTTCCCACAGGCAGCACAG-3’, R: 5’-AATGATGAGAGGCGAAGG-3’, F4/80; F: 5’-GCAAGGAGGACAGATTTATCGTG-3’, R: 5’-GCAAGGAGGACAGAGCTT-3’; IL-1β; F: 5’-CCTGGTCCTGTGAAATGAAACGG-3’, R: 5’-CAGTGCCATACCTTTAGGAAAG-3’; iNOS: F: 5’-GCATGG ACCAGTATAAGCAGGA-3’, R: 5’-CATGG ACCAGTATAAGGCAA GCA - 3’, R: 5’ - GCT TCTGGTCGATGTCATGAGCAA - 3’) to create a standard curve. Abundance of a particular transcript in experimental samples were calculated by comparison to the corresponding standards and normalized to GAPDH transcript levels.

**Nicotinic Acid Treatment of Mice**

WT male mice (9 wk old) were put on either a HF diet and injected with either nicotinic acid (HF-NA) or saline (HF-vehicle) or on a regular chow and saline (RD-
Vehicle) for 30 d. Nicotinic acid was dissolved in sterile normal saline and given in a twice daily dose of 200 µmol/kg body weight; an equal volume of saline was given in the same manner to the control mice (Vehicle). Mice then were fasted at 5:00 p.m. and blood and organs were obtained next morning at 11:00 a.m.

**Wy14,643 Treatment of Mice**

Seven-month old WT male mice were treated with the PPARα agonist Wy14,643 (Biomol International, LP (Plymouth Meeting, PA). The drug was mixed with the powdered diet (regular chow) in a concentration of 0.1 % w/w. Control diet (regular chow) also was powdered in a similar way. Both diets were provided fresh every day for 7 d. After 7 d, the mice were fasted at 5:00 p.m. and whole blood and organs were obtained as above. Liver lysates were used to assess the mRNA levels of CEACAM1, CPT-I, PEPCK, G-6-Pase and β-actin using the specific mouse probes for each. Protein levels of CEACAM1, PPARα, CD36 and tubulin also were analyzed in liver using the specific antibodies against each. Lysates from the WAT were obtained for FAS and FATP-1 protein levels, as well as soleus muscle for FATP-1.

**PPARα Knockout Mice**

PPARα+/+ and PPARα−/− male mice (C57 BL/6J) (6-8 wk old before HF feeding) were purchased from Taconic laboratories and were placed on the HF diet or regular chow for 30 d.
**Fast-Refeed Mice**

WT male mice (4-5 mo old) were fasted at 5:00 p.m. for 24 h. The next day they were allowed to have free access to food for 0, 1, 3, 4, 5, 7, 8, 12, or 24 h before being sacrificed to obtain livers. Controls were randomly fed regular chow before they were sacrificed. Following lysis, 75 μg of protein were analyzed on SDS-PAGE to detect CEACAM1 using α-mCC1, CD36 using α-CD36 or FAS using an α-FAS polyclonal antibody. The blots were reprobed with α-tubulin antibody to normalize per amount of protein. For PPARα, mouse PPARα probe was used to measure the amount of mRNA levels and normalized against β-actin. The bands then were measured using densitometer and plotted as arbitrary units.

**Wy14,643 Treatment of FAO Cells**

FAO rat hepatoma cells were maintained in DMEM (Cellgro) in a 5% CO2 incubator. The media were supplemented with a 10% fetal bovine serum (FBS; Cellgro), 10% heat-inactivated horse serum (Cellgro), 1% penicillin/streptomycin, and 1% glutamine (Invitrogen). The media were changed with fresh ones every 48 h. The cells were grown up to 80% confluency before the media were replaced with fetal bovine serum-dialyzed media (Invitrogen) for 16 h before changing them into the treatment media.

The PPARα agonist (Wy14,643) from (Biomol) was dissolved in ethanol to have a final concentration of 50 μM in each tissue culture plate (10 CC plates). Ethanol was used as a solvent such that its final concentration is 0.1%, or alone as a vehicle in the
control plates of the same concentration. Cells were incubated with the treatment for the corresponding time point then harvested for mRNA or protein analysis of CEACAM1.
RESULTS

Reduced Hepatic Ceacam1 mRNA Levels in Obese Rodents

Fatty rats that exhibit obesity (fa/fa zucker) with diabetes (ZDF) and hypertension (Koletsky) display high fasting plasma FFA and triglyceride in addition to hyperinsulinemia with reduced insulin clearance (as measured by steady-state C-peptide/insulin molar ratio) (Table I). Consistent with the role of CEACAM1 in insulin clearance, which mostly occurs in liver (Poy et al., 2002b), the mRNA levels of CEACAM1-4L Ceacam1 (Ccl) are reduced (by ~50-65%, \( P \lt 0.05 \)) in the liver, but not the kidney of these rats (Table I). As suggested by previous studies in our laboratory on the primary role of impaired insulin clearance in insulin resistance (Poy et al., 2002b), phosphorylation of the insulin receptor \( \beta \)-subunit (IR\( \beta \)) in response to insulin is reduced in these rats (not shown).

High Fat Diet Causes Visceral Obesity and Reduces Hepatic CEACAM1 Levels

To investigate whether obesity is associated with reduced hepatic CEACAM1, we assessed the effect of HF diet on hepatic CEACAM1 levels and insulin action in 4-mo-old C57BL/6J (BL6) male mice. By comparison to RD that derives almost comparable amount of calories from sucrose (14% versus 17%) but less calories from fat (12% versus 45%) and its fat composition was 36.3% saturates; 45.3% monounsaturated
Table I. Reduced Hepatic Ceacam1, impaired insulin clearance and altered fat metabolism in obese rats

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<tr>
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<th>fa/fa lean (fa/+ )</th>
<th>fa/fa obese (fa/fa)</th>
<th>ZDF lean</th>
<th>ZDF obese</th>
<th>Koletsky lean</th>
<th>Koletsky obese</th>
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<td><strong>Younger Ages:</strong></td>
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<tr>
<td>Ceacam1 (mRNA) Liver (x 10^4)</td>
<td>8.98 ± 1.17</td>
<td>4.65 ± 0.50^A</td>
<td>6.96 ± 0.47</td>
<td>5.35 ± 0.34^A</td>
<td>2.58 ± 0.19</td>
<td>1.38 ± 0.16^A</td>
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<tr>
<td>Kidney (x 10^3)</td>
<td>7.47 ± 0.58</td>
<td>6.14 ± 0.44^A</td>
<td>NA</td>
<td>NA</td>
<td>3.15 ± 0.26</td>
<td>3.07 ± 0.35^A</td>
</tr>
<tr>
<td>Insulin (pM) x 10^2</td>
<td>2.95 ± 0.77</td>
<td>18.2 ± 1.59^A</td>
<td>0.23 ± 0.02</td>
<td>4.53 ± 1.92^A</td>
<td>0.60 ± 0.09</td>
<td>12.3 ± 1.15^A</td>
</tr>
<tr>
<td>C-Peptide (pM) x 10^3</td>
<td>1.01 ± 0.20</td>
<td>3.32 ± 0.18^A</td>
<td>0.12 ± 0.20</td>
<td>3.00 ± 0.18^A</td>
<td>0.93 ± 0.13</td>
<td>4.20 ± 0.14^A</td>
</tr>
<tr>
<td>C-Peptide / Insulin</td>
<td>3.80 ± 0.59</td>
<td>1.80 ± 0.08^A</td>
<td>NA</td>
<td>NA</td>
<td>15.8 ± 1.30</td>
<td>3.50 ± 0.30^A</td>
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<tr>
<td>FFA (mEq/l)</td>
<td>0.45 ± 0.01</td>
<td>1.42 ± 0.10^A</td>
<td>0.83 ± 0.06</td>
<td>1.15 ± 0.10^A</td>
<td>0.79 ± 0.20</td>
<td>1.20 ± 0.16^A</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>13.7 ± 2.60</td>
<td>246. ± 41.9^A</td>
<td>72.5 ± 8.00</td>
<td>247. ± 10.5^A</td>
<td>77.8 ± 10.5</td>
<td>343. ± 15.6^A</td>
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<td><strong>Older Ages:</strong></td>
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<tr>
<td>Ceacam1 (mRNA) Liver (x 10^4)</td>
<td>NA</td>
<td>NA</td>
<td>22.9 ± 1.10</td>
<td>9.20 ± 0.61^A</td>
<td>11.0 ± 0.89</td>
<td>5.40 ± 0.26^A</td>
</tr>
<tr>
<td>Insulin (pM) x 10^2</td>
<td>NA</td>
<td>NA</td>
<td>0.87 ± 0.12</td>
<td>7.23 ± 1.00^A</td>
<td>1.03 ± 0.28</td>
<td>25.5 ± 0.58^A</td>
</tr>
<tr>
<td>C-Peptide (pM) x 10^3</td>
<td>NA</td>
<td>NA</td>
<td>1.32 ± 0.05</td>
<td>2.95 ± 0.38^A</td>
<td>1.49 ± 0.40</td>
<td>5.15 ± 0.16^A</td>
</tr>
<tr>
<td>C-Peptide / Insulin</td>
<td>NA</td>
<td>NA</td>
<td>15.7 ± 1.30</td>
<td>5.00 ± 0.50^A</td>
<td>14.6 ± 0.55</td>
<td>2.00 ± 0.05^A</td>
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<tr>
<td>FFA (mEq/l)</td>
<td>NA</td>
<td>NA</td>
<td>0.62 ± 0.07</td>
<td>1.07 ± 0.18^A</td>
<td>0.68 ± 0.10</td>
<td>1.11 ± 0.20^A</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>NA</td>
<td>NA</td>
<td>65.2 ± 2.80</td>
<td>531. ± 57.7^A</td>
<td>112. ± 8.10</td>
<td>385. ± 29.0^A</td>
</tr>
</tbody>
</table>

^A P < 0.05 Obese versus lean. Values are expressed as mean ± S.E. Younger ages: 7-8 wk for fa/+, 6 wk for ZDF and 8 wk for Koletsky. Older ages: 12 wk for ZDF and 16 wk for Koletsky.
and 18.5% polyunsaturated, HF diet caused visceral adiposity with an increasingly more significant effect after 30 d of feeding (Table II). When mice fed HF for 30 d were placed back on RD for 30 d, their adipose fat content was restored (Table II, HF/RD). When normalized per β-actin and α-actin, mRNA (Figure 7A) and protein (Figure 7B) analyses, respectively revealed a significant decrease (about ≥ 60%) in hepatic CEACAM1 levels after 30, but not 9 d of HF diet, and that switching back to regular diet for 30 d (HF/RD) restored CEACAM1. The HF intake for 12-21 d caused a variable effect on CEACAM1 levels ranging between 35-50% reduction (data not shown). This indicates that HF diet reduces hepatic CEACAM1 in a reversible manner.

**HF Diet Leads to Impairment of Insulin Clearance**

Consistent with reduced hepatic CEACAM1, insulin clearance as indicated by C-peptide/insulin molar ratio, was impaired ($P<0.05$) in mice fed HF diet for 30, but not 9 d (Table II). Accordingly, HF for 30 d caused hyperinsulinemia ($P<0.05$) (Table II). Switching back to RD after 30 d of HF restored insulin clearance and insulin levels (Table II).
Table II. The effect of High-fat Diet on The Metabolic Phenotype

<table>
<thead>
<tr>
<th></th>
<th>9 Days</th>
<th>30 Days</th>
<th>30/30</th>
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<tr>
<td></td>
<td>RD</td>
<td>HF</td>
<td>RD/RD</td>
</tr>
<tr>
<td>% Visceral Fat/BWT</td>
<td>1.15 ± 0.13</td>
<td>1.92 ± 0.10&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.41 ± 0.25</td>
</tr>
<tr>
<td>Plasma FFA (mEq/l)</td>
<td>0.47 ± 0.06</td>
<td>0.66 ± 0.05</td>
<td>0.49 ± 0.11</td>
</tr>
<tr>
<td>Plasma TG (mg/dl)</td>
<td>18.0 ± 3.31</td>
<td>26.5 ± 4.84</td>
<td>17.2 ± 1.68</td>
</tr>
<tr>
<td>Hepatic TG (µg/mg)</td>
<td>273. ± 52.2</td>
<td>375. ± 84.2</td>
<td>166. ± 21.1</td>
</tr>
<tr>
<td>Plasma Insulin (pM)</td>
<td>362. ± 7.20</td>
<td>422. ± 24.5</td>
<td>431. ± 47.7</td>
</tr>
<tr>
<td>Plasma C-Peptide (pM)</td>
<td>235. ± 35.6</td>
<td>346. ± 43.1</td>
<td>323. ± 42.2</td>
</tr>
<tr>
<td>C-Peptide/Insulin</td>
<td>0.81 ± 0.17</td>
<td>0.77 ± 0.12</td>
<td>0.87 ± 0.10</td>
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<tr>
<td>Fasting Glucose (mg/dl)</td>
<td>100. ± 2.10</td>
<td>104. ± 5.60</td>
<td>96.5 ± 5.90</td>
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<tr>
<td>Random Glucose (mg/dl)</td>
<td>NA</td>
<td>NA</td>
<td>103. ± 4.41</td>
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<sup>A</sup> P < 0.05 HF versus RD. n= 8 animals per category. Values are expressed as mean ± S.E.
Figure 7. **High-fat Intake for 30 d Decreases Hepatic CEACAM1 in Normal WT Mice**

Normal WT male mice (4 mo old) were fed for 9-30 d either a regular chow (RD) or a HF diet. Following HF for 30 d, some were placed back on RD for 30 d (HF/RD). Liver lysates were analyzed by northern (Panel A) and western blot (Panel B) analyses. The mRNA and proteins were probed with β-actin cDNA (A) and α-actin antibody (B) to normalize for the amount of CEACAM1 added. Autoradiograms (A) and ECL gels (B) were scanned and density of CEACAM1 was normalized per the density of actin and plotted in the graph on the right. Two out of 6 mice from each category (1 and 2) are shown. Northern (Figure 7A) and Western (Figure 7B) analyses revealed a marked decrease (~by ≥ 60%) in hepatic CEACAM1 levels after 30, but not 9 d of HF diet, and that switching back to regular diet for 30 d (HF/RD) restored CEACAM1 levels. HF intake for 12-21 d caused a variable effect on CEACAM1 levels ranging between 35-50% reduction (not shown). Thus, HF diet reduced hepatic CEACAM1 in a reversible manner. (n=8 animals per category). Values are expressed as mean ± SE. *P<0.05.
**HF-induced Insulin Resistance**

Subjecting liver lysates to phosphorylation in the presence of insulin prior to immunoprecipitation (IP) with α-IRβ and immunoblotting with α-phosphotyrosine (α-pTyr) antibodies revealed that IR phosphorylation was reduced following 30 d of HF intake (Figure 8A, orange versus gray bar), but was intact in mice fed HF for 9 d. Switching the diet to RD restored insulin-induced IR phosphorylation (Figure 8A, 30HF/30RD versus RD). Moreover, mice on HF diet for 30, but not 9 d, exhibited glucose intolerance (Figure 8B), which was reversed upon refeeding with RD [(Figure 8B, Panel 30/30 (RD)]. Moreover, glucose uptake, assessed by Western analysis (immunoblotting, Ib) of phosphorylated Akt per total Akt in WAT lysates, was markedly reduced in white adipose tissue (WAT) and soleus muscle of mice fed HF for 30 d (Figure 8C). Ex-vivo phosphorylation of IR in WAT (Figure 8C) and muscle lysates (Figure 8C) revealed intact receptor activation by insulin, as suggested by similar increase in phosphotyrosine incorporation into IRβ in the presence of insulin in HF- and RD-fed mice (Figure 8C). This suggests that HF diet did not alter intrinsic insulin action in WAT as it did in liver, and that the decrease in glucose transport is a manifestation of increased lipid flux from liver to muscle, as has been reported in BL6 mice on HF diet (de Fourmestraux et al., 2004; Park et al., 2005). In light of concomitant decrease in insulin removal, it is conceivable that insulin resistance develops initially in the liver in response to fat feeding. Consistent with insulin resistance, HF intake for 30 d increased random blood glucose (Table II); however, it did not significantly alter fasting blood glucose (Table II), suggesting insulin resistance without frank diabetes. This probably
results from normal β–cell function and elevation in compensatory insulin secretion, as suggested by higher C-peptide level in 30-d-HF-fed mice (549.0 ± 56.7 pM in HF- versus 323.0 ± 42.2 in RD, \( P<0.05 \)) (Table II).
Figure 8.  High-fat Intake for 30 d Induces Insulin Resistance

(A) High-fat intake for 30 d reduces hepatic (IR) phosphorylation in a reversible manner. Lysates from liver were treated either buffer (-) or 100 µM insulin (+) in the presence of ATP. The insulin receptor (IRβ) was immunoprecipitated (IP) with monoclonal antibody (α-IRβ) and analyzed by 7% SDS-PAGE followed by immunoblotting (Ib) with α-phosphotyrosine (α-pTyr) to assess phosphorylation. Proteins were re-immunoblotted (reIb) with α-IRβ to normalize by the amount of IR added. Gels were scanned and the band density of phosphorylated proteins was divided that of IR and represented in the graph. Gels are representative of three mice per category (*P<0.05).

(B) Glucose tolerance (IPGTT) in mice after 30 d of HF feeding. Mice were fed a HF-diet for 9-30 d and challenged with an intra-peritoneal glucose injection (2 mg/kg body weight) to assess glucose tolerance at times 0, 15, 30, 60 and 120 min after the injection. Mice on HF diet for 30 d, but not on 9 d, exhibited glucose intolerance, which was reversed upon refeeding with RD for 30 d (Panel 30/30 RD).

(C) IR and Akt phosphorylation in WAT and soleus muscle. In response to HF feeding for 30 d, western analyses revealed normal IR phosphorylation in WAT and soleus muscle. However, phosphorylated Akt per total Akt in WAT lysates was markedly reduced in WAT and soleus muscle.
A.  

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<tr>
<td>9 Days</td>
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</tr>
<tr>
<td>IP: α-IR_β</td>
<td>+</td>
<td>+</td>
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<tr>
<td>lb: α-pTyr</td>
<td>+</td>
<td>+</td>
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<td>relb: α-IR_β</td>
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30 Days  

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30/30 Days  

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B.  

Blood Glucose (mg/dl)  

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C.  

**WAT**  

**IR Phosphorylation:**  

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**Akt Phosphorylation:**  

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**Soleus Muscle**  

**IR Phosphorylation:**  

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**Akt Phosphorylation:**  

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</tr>
<tr>
<td>relb: α-Akt</td>
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</table>
Increased Mobilization of FFA, but not Cytokines from the Adipose Tissue of Mice

Consistent with hyperinsulinemia, hepatic FAS level was markedly elevated in mice fed HF for 30 d (Figure 9A). This contributes to increased lipogenesis and hepatic triglyceride content (Table II). Despite increased lipogenesis, plasma triglyceride level was normal (Table II), which could be due to low triglyceride output from liver and/or enhanced uptake and hydrolysis in muscle and fat tissue. Elevated protein levels of FATP-1, a major fatty acid transporter in muscle and WAT (Figure 9B & C, respectively), together with reduced glucose transport (Park et al., 2005), supports the latter.

Nonetheless, fasting plasma FFA increased significantly ($P<0.05$) after 30, but not 9 d of HF intake, and it was restored after switching to RD (Table II). Together, the data suggest that high plasma FFA resulted from increased output from WAT, in agreement with reports on the effect of fat-feeding on insulin clearance in dogs (Kabir et al., 2005).

Quantitative Real Time PCR analysis revealed normal mRNA levels of fat-associated macrophage markers (CD68: 0.160 ± 0.08 molecules/GAPDH in HF versus 0.900 ± 0.17 in RD and F4/80: 0.165 ±0.02 in HF versus 0.575 ± 0.11 in RD) and adipokines (iNOS: 0.097 ± 0.01 in HF versus 0.120 ± 0.02 in RD). The RIA assay also revealed intact plasma leptin levels in WT mice fed HF for 30 d (3.611 ± 0.642 ng/ml versus 2.763 ± 0.187 ng/ml in RD). In agreement with reports on delayed elevation of adipokine levels in mice fed a high fat diet (Xu et al., 2003), our data exclude a role for adipokines in insulin resistance induced by HF feeding for 30 d, and implicate WAT-derived plasma FFA in the process.
Figure 9. Elevated Levels of Proteins Involved in Fat Transport and Synthesis after 30 d of HF Intake

(A) Liver lysates were analyzed by western blot analyses using α-FAS and α-CD36 antibody, then were re-immunoblotted with α-actin antibody normalize for the amount of the protein added.

(B) Lysates from soleus muscle were analyzed by western blot analyses using α-FATP-1 antibody, then were re-immunoblotted with α-actin antibody to normalize for the amount of the protein added.

(C) WAT lysates were analyzed by western blot analyses using α-FATP-1, then were re-immunoblotted with α-actin antibody normalize for the amount of the protein added.
Inhibition of Lipolysis from Visceral Adipose Tissue by Nicotinic Acid Blunts the Deleterious Effect of HF on Hepatic CEACAM1 and Insulin Action.

In order to further confirm the role of FFA on hepatic CEACAM1 levels in vivo, we inhibited lipolysis in 30-d HF-fed 3-mo-old male BL6 mice with nicotinic acid. As expected, after 30 d of HF feeding, mice developed visceral obesity as expressed by weight of visceral fat divided by total body weight in both HF-fed groups (HF-Veh, and HF-NA) (Table III) compared to RD and vehicle treated (RD-Veh) control group (P<0.05). In consistent with the development of visceral obesity in the HF-Veh group, their fasting plasma FFA level was elevated (Table III), (P<0.05). However, when we injected the HF-fed mice with a twice daily dose of nicotinic acid, lipolysis was inhibited as indicated by fasting plasma FFA levels in the HF-NA group which was restored to normal values that are comparable to those of the RD-Veh (Table III). Fasting plasma triglyceride values were not changed in the three groups after 30 d of HF or RD feeding with or without nicotinic acid, although there was some slight (statistically insignificant) increase in the HF-Veh group (Table III).

As shown before, 30 d of HF feeding in mice developed visceral adiposity and increased fasting plasma FFA levels and caused reduction in hepatic CEACAM1 levels (Figure 7) as a result of FFA release from adipose tissue. Interestingly, when we inhibited the release of FFA from adipose tissue by nicotinic acid, hepatic CEACAM1 levels were normal as shown in (Figure 10A). The preservation of hepatic CEACAM1 levels after inhibition of lipolysis also was evident as we observed normal insulin clearance in the HF-NA treated mice (Table III) compared to impaired insulin clearance (P<0.05) in the
HF-Veh (Table III). Accordingly, HF for 30 d caused hyperinsulinemia (P<0.05) in the HF-Veh group, but not in the HF-NA (Table III). All three groups showed similar insulin secretion as indicated by C-Peptide levels (Table III).

When we inhibited lipolysis by nicotinic acid, we prevented the release of FFA and their elevation in the circulation, therefore, we did not notice a decrease in hepatic CEACAM1, and hence insulin resistance did not also develop in these mice as indicated by random hyperglycemia (P<0.05) (Table III). On the other hand, all groups exhibited normal fasting blood glucose levels (Table III) which means there is no frank diabetes. Taken together these data indicate that insulin resistance did not develop when reduction of hepatic CEACAM1 by FFA was prevented via nicotinic acid-induced suppression of lipolysis.

**PPARα Activation Decreases CEACAM1 Levels**

The rise in fasting plasma FFA prompted us to examine PPARα activation in HF fed-mice and to investigate whether this could cause reduction in CEACAM1 levels. The HF intake increased fasting hepatic PPARα mRNA even after 9 d, but did not alter the protein content until after 30 d (~3- to 4-fold) (Figure 11). Switching to RD restored PPARα mRNA and proteins (Figure 11). Consistent with increased PPARα activation in mice fed HF for 30 d, hepatic CD36, a target of PPARα, was elevated (Figure 9A). This suggests an inverse relationship between PPARα activation and hepatic CEACAM1.
Table III. The Effect of Nicotinic Acid Treatment on The Metabolic Phenotype of HF diet-fed Mice

<table>
<thead>
<tr>
<th></th>
<th>RD-Veh</th>
<th>HF-Veh</th>
<th>HF-NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Visceral Fat/BWT</td>
<td>1.26 ± 0.09</td>
<td>2.74 ± 0.28$^A$</td>
<td>2.28 ± 0.22$^A$</td>
</tr>
<tr>
<td>Plasma FFA (mEq/l)</td>
<td>0.60 ± 0.06</td>
<td>0.92 ± 0.11$^A$</td>
<td>0.68 ± 0.04</td>
</tr>
<tr>
<td>Plasma TG (mg/dl)</td>
<td>109. ± 9.69</td>
<td>134. ± 12.2</td>
<td>120. ± 12.8</td>
</tr>
<tr>
<td>Plasma Insulin (pM)</td>
<td>64.4 ± 5.83</td>
<td>89.0 ± 7.95$^A$</td>
<td>62.2 ± 4.97</td>
</tr>
<tr>
<td>Plasma C-peptide (pM)</td>
<td>475. ± 97.8</td>
<td>594. ± 123.</td>
<td>648. ± 114.</td>
</tr>
<tr>
<td>C-Peptide/Insulin</td>
<td>6.01 ± 0.37</td>
<td>4.79 ± 0.44$^A$</td>
<td>7.05 ± 0.42</td>
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<tr>
<td>Fasting Glucose (mg/dl)</td>
<td>101. ± 4.08</td>
<td>98.9 ± 7.81</td>
<td>99.1 ± 12.2</td>
</tr>
<tr>
<td>Random Glucose (mg/dl)</td>
<td>144. ± 3.31</td>
<td>156. ± 3.73$^A$</td>
<td>140. ± 2.07</td>
</tr>
</tbody>
</table>

RD-Veh: Regular diet and vehicle treated mice, HF-Veh: HF diet and vehicle, HF-NA: HF diet and Nicotinic acid treated mice. $^A P <0.05$. (n=10 mice per category). Values are expressed as mean ± SE.
Figure 10. Inhibition of Lipolysis by Nicotinic Acid Prevents HF-diet-Induced Reduction of Hepatic CEACAM1

Liver lysates from WT mice after 30 d of either a regular chow (RD) or a HF were treated simultaneously with either a vehicle (Veh) or a nicotinic acid injection (NA) were analyzed by western blot analyses using α-CC1 antibody, then were re-immunoblotted with α-actin antibody to normalize for the amount of the protein added.
Figure 11. **High-fat Intake for 30 d Increases Hepatic PPARα Levels**

Normal WT male mice (4 mo old) were fed for 9-30 d either a regular chow (RD) or a high-fat diet (HF). Following HF for 30 d, some were placed back on RD for 30 d (HF/RD). Liver lysates were analyzed by northern (Panel A) and western blot (Panel B) analyses. mRNA and proteins were probed with β-actin cDNA (A) or α-actin antibody (B) to normalize for the amount of PPARα added. Autoradiograms (A) and ECL gels (B) were scanned and density of PPARα was normalized per the density of actin and plotted in the graph on the right. Two out of 6 mice from each category (1 and 2) are shown. (*P<0.05). Values are expressed as mean ± SE.
To test whether PPARα activation at fasting regulates hepatic CEACAM1 content, we treated 7 mo-old mice with the PPARα agonist, Wy14,643 (Wy) (0.1% wt/wt) for 7 d. As expected, this increased fasting hepatic PPARα (and its targets, CD36/FABP and CPT1) (Figure 12A and B). It also increased the mRNA levels of PEPCK and glucose-6-phosphatase (G-6-Pase) (Figure 12A), to promote gluconeogenesis at fasting. Accordingly, fasting glucose level was normal in Wy-treated mice (Table IV). Additionally, PPARα activation also decreased fat mass (Table IV) and lipogenesis as indicated by the reduction in hepatic triglyceride content (Table IV). This may contribute to reduced insulin secretion, as measured by C-peptide levels (Table IV).

In contrast to its positive effect on fatty acid oxidation and gluconeogenesis, PPARα activation markedly reduced hepatic CEACAM1 mRNA (Figure 12A) and protein content (Figure 12B) and consequently, impaired insulin clearance as measured by the molar ratio C-peptide/insulin (Table IV). This could occur to compensate for decreased insulin secretion, limit insulin deficiency (mild decreased insulin levels, Table IV), prevent hyperglycemia, and maintain normal insulin action in liver and muscle, as suggested by intact insulin-induced tyrosine phosphorylation of IRβ in both tissues (Figure 13A and B).
Figure 12. PPARα Activation by Wy14,643 Decreases Hepatic CAECAM1 Levels and Lipogenesis

WT male mice (7-mo-old) were treated with either the PPARα agonist, Wy14,643 (Wy) which was mixed as 0.1% wt/wt in regular chow (Wy) or a regular chow alone (RD) for 7 d. Panel (A), Northern blotting showing mRNA levels of Ceacam1, CPT-1, PEPCK and G-6-Pase using probes specific for Ceacam1, CPT-1, PEPCK and G-6-Pase, respectively. Panel (B), Western blot analyses showing the protein levels of CAECAM1, PPARα and its target CD36 as a positive control.

A.  B.

<table>
<thead>
<tr>
<th>RD</th>
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<tbody>
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<tr>
<td>2</td>
<td>2</td>
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\[\begin{array}{c}
\text{Cc1} \\
\text{CPT-I} \\
\text{PEPCK} \\
\text{G-6-Pase} \\
\text{β-Actin}
\end{array}\]

\[\begin{array}{c}
\text{lb: } \alpha\text{-CC1} \\
\text{lb: } \alpha\text{-PPARα} \\
\text{lb: } \alpha\text{-CD36} \\
\text{relb: } \alpha\text{-Actin}
\end{array}\]

\[\begin{array}{c}
\text{CC1} \\
\text{PPARα} \\
\text{CD36} \\
\text{Actin}
\end{array}\]
Table IV. Effect of PPARα Activation by Wy14,643 on the Metabolic Phenotype of WT Male Mice

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<th>Chow Diet</th>
<th>Wy-Diet</th>
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<td>% Visceral Fat/BWT</td>
<td>5.44 ± 1.65</td>
<td>0.49 ± 0.05A</td>
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<tr>
<td>Plasma FFA (mEq/l)</td>
<td>0.37 ± 0.04</td>
<td>0.39 ± 0.05</td>
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<td>Plasma TG (mg/dl)</td>
<td>70.3 ± 8.15</td>
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<td>Hepatic TG (µg/mg protein)</td>
<td>12.3 ± 0.88</td>
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<td>Plasma Insulin (pM)</td>
<td>464. ± 18.9</td>
<td>354. ± 10.6A</td>
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<tr>
<td>Plasma C-peptide (pM)</td>
<td>488. ± 95.3</td>
<td>99.2 ± 11.8A</td>
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<td>C-Peptide/Insulin</td>
<td>1.04 ± 0.19</td>
<td>0.28 ± 0.03A</td>
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<td>Fasting Glucose (mg/dl)</td>
<td>90.3 ± 11.1</td>
<td>82.0 ± 8.77</td>
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^P<0.05 Wy- vs Chow diet. (n=8-9 mice per group).

Values are expressed as mean ± SE.
Figure 13. IR Phosphorylation in Liver and Soleus Muscle of WT Mice Treated with Wy14,643

Lysates from liver (A) or soleus muscle (B) from male mice after 7 d of either a regular chow diet (RD) or a Wy14,643 (Wy) were treated either buffer (-) or 100 μM insulin (+) in the presence of ATP. The insulin receptor (IR<sub>β</sub>) was immunoprecipitated (IP) with a monoclonal antibody (α-IR<sub>β</sub>) and analyzed by 7% SDS-PAGE followed by immunoblotting (Ib) with α-phosphotyrosine (α-Tyr) antibody to assess phosphorylation. For the amount of IR added.

**A. Liver**

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**B. Soleus Muscle**

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<tr>
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<tr>
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To further assess the role of PPARα in the decrease of CEACAM1 levels in mice fed a HF for 30 d, PPARα−/− mice from the BL6 genetic background were fed HF diet for 30 d prior to examining the level of CEACAM1 levels. Despite the increase in visceral obesity and fasting plasma FFA levels in PPARα−/− mice by HF intake (Table V), hepatic CEACAM1 mRNA and protein levels were not altered as in PPARα+/+ mice (Figure 14A i and ii). Consistently, insulin and C-peptide levels were intact (Table V). Moreover, PPARα−/− mice were protected from developing insulin resistance, as indicated by normal random blood glucose, normal insulin levels (Table V) and normal glucose tolerance (Figure 14B). This also was evident by normal insulin-induced IRβ phosphorylation in liver lysates (Figure 14C). Thus, null mutation of PPARα prevented reduction of hepatic CEACAM1 levels and the development of insulin resistance by HF diet. This is consistent with other previous reports that demonstrated that PPARα activation mediates insulin resistance induced by HF intake, and that its deletion protects against the development of insulin resistance (Kersten et al., 1999; Leone et al., 1999; Patsouris et al., 2006).

**Regulation of CEACAM1 Expression by PPARα in Rat Hepatoma Cells**

The PPARα, a transcriptional regulator, may regulate CEACAM1 expression either directly or indirectly via regulating other genes, which may induce metabolic changes that in turn, affect CEACAM1 content. To address this issue, we treated rat hepatoma FAO cells with PPARα agonist, Wy14,643, for 0-24 h. As Figure 15 reveals,
Table v. The effect of 30 days High-fat Diet on the Metabolic Phenotype of PPARα<sup>+</sup>/+ and PPARα<sup>-/-</sup> Mice

<table>
<thead>
<tr>
<th></th>
<th>PPARα&lt;sup&gt;+&lt;/sup&gt;</th>
<th>PPARα&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td>% Visceral Fat/BWT</td>
<td>1.30 ± 0.10</td>
<td>5.09 ± 0.17&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>Plasma FFA (mEq/l)</td>
<td>0.72 ± 0.06</td>
<td>1.10 ± 0.07&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma TG (mg/dl)</td>
<td>137. ± 8.57</td>
<td>116. ± 10.5</td>
</tr>
<tr>
<td>Hepatic TG (µg/mg protein)</td>
<td>41.1 ± 3.44</td>
<td>58.6 ± 3.82&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>Muscle TG (µg/mg protein)</td>
<td>31.3 ± 5.76</td>
<td>59.2 ± 9.40&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma Insulin (pM)</td>
<td>459. ± 84.5</td>
<td>707. ± 122&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>Plasma C-Peptide (pM)</td>
<td>323. ± 42.2</td>
<td>549. ± 56.7&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>C-Peptide/Insulin</td>
<td>1.05 ± 0.10</td>
<td>0.81 ± 0.05&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>Fasting Glucose (mg/dl)</td>
<td>114. ± 3.85</td>
<td>119. ± 5.17</td>
</tr>
<tr>
<td>Random Glucose (mg/dl)</td>
<td>125. ± 3.15</td>
<td>150. ± 1.94&lt;sup&gt;B&lt;/sup&gt;</td>
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</table>

<sup>A</sup><sup>P</sup>&lt;0.05 PPARα<sup>-/-</sup>-RD vs PPARα<sup>+</sup>/+RD, <sup>B</sup><sup>P</sup>&lt;0.05 HF vs RD. Values are expressed as mean ± SE.
Figure 14. PPARα Null Mice (PPARα\(^{-/-}\)) are protected against HF-diet-induced Reduction in Hepatic CEACAM1 and Insulin Resistance

(A) PPARα\(^{+/+}\) male mice and their PPARα\(^{-/-}\) counterparts (4-mo-old) were fed for 30 d either RD or HF diet. Liver lysates were analyzed by northern (A \(i\)) and western blot (A \(ii\)) analyses using Ceacam1 specific probe (A \(i\)) or α-CC1 antibody (A \(ii\)). mRNA and proteins were then re-probed with β-actin cDNA (A \(i\)) or α–actin (A \(ii\)) to normalize for the amount of CEACAM1 added.

(B) Impaired intra-peritoneal glucose tolerance test (IPGTT) in PPARα\(^{+/+}\) mice after 30 d of HF feeding (right panel) and normal glucose tolerance in PPARα\(^{-/-}\) (left panel). Mice were fed a HF-diet for 30 d and challenged with an intra-peritoneal glucose injection (2 mg/kg body weight) to assess glucose tolerance at times 0, 15, 30, 60 and 120 min after the injection. PPARα\(^{+/+}\) mice on HF diet for 30 days, but not PPARα\(^{-/-}\), exhibited glucose intolerance.

(C) Liver lysates from PPARα\(^{+/+}\) (left panel) and PPARα\(^{-/-}\) (right panel) after 30 d of either a RD or HF were treated either buffer (-) or 100 µM insulin (+) in the presence of ATP. The insulin receptor (IRβ) was immunoprecipitated (IP) with a monoclonal antibody (α-IRβ) and analyzed by 7% SDS-PAGE followed by immunoblotting (Ib) with α-phosphotyrosine (α-pTyr) antibody to assess phosphorylation. Proteins
then were re-immunoblotted (reIB) with $\alpha$-IR$_{\beta}$ to normalize for the amount of IR added.

A. CEACAM1

\[ \text{mRNA} \]

\[ \text{Protein} \]

B. IPGTT

\[ \text{Blood Glucose (mg/dl)} \]

\[ \text{Post Glucose (Min)} \]

C. IR-Phosphorylation

\[ \text{IP: } \alpha$-IR$_{\beta}$ \]

\[ \text{lb: } \alpha$-$p$Tyr \]

\[ \text{relb: } \alpha$-IR$_{\beta}$ \]
Figure 15. **PPARα Activation by Wy14,643 Decreases CEACAM1 Levels in Rat Hepatoma (FAO) Cells**

Cellular lysates from rat hepatoma (FAO) cells were treated with either PPARα agonist (Wy14,643; the Wy or (+) lane) or vehicle (the (EtOH) or (-) lane) for different time points (0.5-24 h) and then were analyzed by northern (Figure 15A) and western (Figure 15B) analyses. MRNA and proteins were re-probed with GAPDH cDNA or α-tubulin antibody to normalize for the amount of CEACAM1 added. Autoradiograms (A) and ECL gels (B) were scanned and density of CEACAM1 was normalized per the density of GAPDH or tubulin and plotted in the graph underneath.
Activation of PPARα progressively decreased CEACAM1 mRNA and protein content beginning at 1 and 2 h, respectively (orange versus gray bars). This rapid negative effect of PPARα activation on Ceacam1 mRNA levels suggests that PPARα directly regulates Ceacam1, and that this may occur at the transcriptional level. More experiments are needed to test and characterize this novel active repressing effect by PPARα.

**CEACAM1 is regulated by Fasting/Refeeding**

Because hepatic PPARα is mainly activated at the fasting state when the liver becomes the “dumping sink” of mobilized FFA, we examined whether CEACAM1 is regulated by fasting/refeeding. Wild-type mice were fasted for 24 h (F) and refed for 1-24 h. The level of hepatic proteins was compared to the randomly fed state (R). As expected, the level of PPARα (Figure 16A) and its target, CD36 (Figure 16B), was highest at fasting and gradually decreased during refeeding. In contrast, CEACAM1 levels were low at fasting and peak at 4 and 7-8 h of refeeding. The peaks of CEACAM1 levels corresponded with pulses of insulin secretion at 1, 4 and 7 h of refeeding (Najjar et al., 2005). On the other hand FAS levels remained low in the first few hours of refeeding and continued to progressively increase until 24 h of refeeding (Figure 16A). This suggests that low FAS activity during the first few hours of refeeding is mediated by the higher ratio of CEACAM1 relative to FAS until about 8 h of refeeding, at which point, the raio of FAS to CEACAM1 is reversed (Figure 16A) and FAS is relieved from the negative pressure of insulin via a CEACAM1-dependent mechanism.
Figure 16.  **CEACAM1 Regulation by Fasting/Refeeding**

After a 24 h fasting (F), normal WT mice were fed for 1-24 h (Fast-Refeed) or allowed to have free access to food all the time [Random (R)]. Then we analyzed liver samples for CEACAM1, PPARα (A) and its target CD36 (B). Liver lysates were analyzed by western (CEACAM1 and FAS) or northern (PPARα). CEACAM1 and FAS proteins were probed with α CEACAM1 and α-FAS antibodies, respectively, and then the blots were re-immunoblotted with α-tubulin antibody to normalize per amount of protein added. mRNA of PPARα was probed with α-PPARα c-DNA then re-probed with α-actin for normalization. The density of each band was measured and divided by the density of tubulin (for protein) or β-actin (for mRNA) and plotted on an arbitrary scale (lower panel).
HF intake for 30 days did not alter Hepatic PTP-1B Levels

Because the phosphatase PTP-1B terminates insulin signal and causes insulin resistance (Elchebly et al., 1999; Klaman et al., 2000; Zabolotny et al., 2004), we investigated whether it is involved in the development of insulin resistance in HF-fed mice. As Figure 17 reveals, HF intake even for 30 d did not alter hepatic PTP-1B content. This suggests that the insulin resistance that developed after 30 d of HF intake does not involve changes in PTP-1B levels.
Figure 17. High-fat Feeding for 30 d Did Not Alter Hepatic PTP-1B Levels

Normal WT male mice (4 mo old) were fed for 9-30 d either a regular chow (RD) or a (HF) diet. Following HF for 30 d, some were placed back on RD for 30 d (HF/RD). Liver lysates were analyzed by western blot analysis. The blots were probed with a α-PTP-1B antibody then re-immunoblotted with α-actin antibody to normalize per amount of protein added. ECL gels were scanned and density of PTP-1B was normalized per the density of actin and plotted in the graph on the right. Two out of 6 mice from each category (1 and 2) are shown. Values are expressed as mean ± SE.
DISCUSSION

Insulin resistance is the hallmark of T2DM and obesity. In addition to manifestation of an obesity-dependent pro-inflammatory state, insulin resistance is commonly associated with increased FFA mobilization out of the adipose tissue. The increase in plasma FFA may develop before fasting hyperglycemia (Baldeweg et al., 2000; Perseghin et al., 1997; Reaven et al., 1988) and reduction in β-cell function (Kashyap et al., 2003; Krebs and Roden, 2004). Consistently, we herein report that obese rats (fa/la, ZDF and Koletsky) exhibit hyperinsulinemia and insulin resistance in addition to marked alteration in lipid metabolism, including elevation in plasma FFA and triglycerides (Table V). We show that hyperinsulinemia in these rats is associated with impaired insulin clearance (Table V) as well as increased insulin secretion (as measured by C-peptide levels). Consistent with the role of CEACAM1 in promoting insulin clearance (Poy et al., 2002b), which occurs mostly in liver and to a lower extent in kidney, these obese rats exhibit a decrease in Ceacam1 mRNA levels in liver, but not kidney (Table V). Similarly to rats, obese humans exhibit a marked decrease in hepatic CEACAM1 protein content by comparison to their lean counterparts (not shown). Thus, our studies draw a correlation between reduced hepatic CEACAM1 levels and visceral obesity in rodents and humans.

We also show that diet-induced insulin resistance (hyperinsulinemia, glucose intolerance, random hyperglycemia and insulin receptor phosphorylation) develops in parallel to a concomitant decrease in hepatic CEACAM1 levels by >50% and resulting impairment of insulin clearance. This gene-dosage effect is supported by our
observation of reduced insulin clearance and elevated plasma FFA together with obesity and random hyperglycemia in \( p/p \) homozygous mice with partial (~85%) deletion of \( Cc1 \) (Blau et al., 2001), but not in heterozygous \( +/p \) mice with ~ 40% reduction of hepatic \( Cc1 \) mRNA levels (not shown). Impairment of insulin clearance in response to high fat feeding has been shown in dogs (Kabir et al., 2005). However, the observation that this may occur via a decrease in hepatic CEACAM1 levels assigns a critical role for a CEACAM1-dependent pathway in the early pathogenesis of diet-induced visceral obesity and insulin resistance.

Increased fat intake induces insulin resistance in rats (Oakes et al., 1997) and humans (Agus et al., 2000; Bachmann et al., 2001). With acute lipid infusion causing insulin resistance \emph{in vivo} (Krebs et al., 2002; Krebs and Roden, 2004; Roden et al., 1996), it is likely that diet-induced insulin resistance is accounted for, at least partly, by elevation in plasma FFA levels, as has been demonstrated in obese non-diabetic subjects (Boden and Carnell, 2003). Interestingly, we herein show that mice from the C57BL/6 genotype develop insulin resistance in response to high-fat intake in parallel to a >50% decrease in hepatic CEACAM1 levels, which occurred during high fat feeding for 21-30 days. The decrease in CEACAM1 levels is correlated with increased visceral obesity and plasma FFA output. During this short period of fat intake (9-30 days), the gradual increase in visceral obesity and its corresponding increase in FFA output occurred without a significant change in the pro-inflammatory state of these mice, as indicated by normal mRNA levels of macrophage markers (CD68 and F4/80) and adipokines in adipose tissue (TNF-\( \alpha \); IL-6 and IL-1\( \beta \)), and plasma levels of MCP-1, IL-6 and tPAI-1.
Of interest, even a longer period of high fat intake for 60 days failed to increase macrophage recruitment and adipose tissue-associated mRNA levels of IL-6, IL-1β and TNF-α in these C56BL/6 mice, despite the larger increase in visceral obesity and ~10-fold increase in plasma leptin levels (data not shown). These findings are in agreement with the observed delayed adipokine secretion in mice fed a high fat diet (Xu et al., 2003), and with the modest increase of the Monocyte Chemoattractant Protein-1 (MCP-1) after 4 weeks of HF feeding, even when the diet derived 60% of calories from fat (Chen et al., 2005).

That plasma FFA play an important role in the decrease in hepatic CEACAM1 levels and insulin resistance, which are caused by high fat intake, is supported by restoration of CEACAM1 levels together with insulin clearance and action in mice in which plasma FFA levels were reduced to normal by switching to regular diet for 30 days or upon treatment with nicotinic acid. Whereas the former treatment reduces visceral obesity, the latter limits lipolysis without a significant change in visceral obesity (Tunaru et al., 2003). This confirms that FFA play a major role in diet-induced insulin resistance, and that other obesity-related factors, such as adipokines, play a relatively minor role in CEACAM1-dependent pathways, which are involved in diet-induced insulin resistance.

The conclusion that increased visceral obesity and output of FFA contribute significantly to CEACAM1-dependent insulin resistance is in agreement with our previous observations in L-SACC1 mice, with liver-specific inactivation of CEACAM1 (Park et al., 2006). We have also shown that reducing visceral obesity and lipolysis with
carnitine and nicotinic acid, respectively, improved insulin action in L-SACC1 mice (Dai et al., 2004).

Consistent with hyperinsulinemia, hepatic FAS level was markedly elevated in mice fed HF for 30 days (Figure 9A), in agreement with other reports (Escher et al., 2001; Kersten et al., 1999; Leone et al., 1999). This contributes to increased lipogenesis and hepatic triglyceride content (Table II). Despite increased lipogenesis, plasma triglyceride level was normal, which could be due to low triglyceride output from liver and/or enhanced uptake and hydrolysis in muscle and fat tissue. Elevated protein levels of FATP-1, a major fatty acid transporter, in muscle and WAT (Figures 9B and C, respectively), suggest increased fat redistribution to these tissues. In WAT, this is facilitated by increased visceral adiposity, which leads to lipolysis. Of interest, this appears to occur without a significant change in intrinsic insulin action in this tissue in terms of glucose transport, as suggested by intact ex vivo phosphorylation of the insulin receptor and Akt in response to insulin. In contrast, insulin’s ability to activate the insulin receptor and the Akt pathway mediating glucose transport is diminished in the skeletal muscle of mice fed a high fat diet for 30 days. These data are supported by observations of decreased glucose uptake and insulin resistance in muscle of fat-fed mice resulting from lipid redistribution and elevated lipogenesis (de Fourmestraux et al., 2004; Park et al., 2005)

Taken together, this is consistent with a paradigm of high fat intake increasing intra-abdominal adipose tissue deposition and supply of FFA, which causes reduction of hepatic CEACAM1 levels and hepatic insulin resistance, as manifested by increased
reesterification and hepatic triglyceride levels. This, in turn triggers compensatory increase in insulin secretion and triglyceride transport into the adipose tissue, contributing to visceral adiposity, and skeletal muscle where, by competing with glucose uptake, can cause insulin resistance.

Mechanistically, we have observed that high fat diet causes a reduction of hepatic CEACAM1 mRNA and protein levels via activation of PPARα, which is known to upregulate genes involved in generating energy and the reduction potential from fatty acid β-oxidation in the mitochondria to support gluconeogenesis at fasting (Pegorier et al., 2004). This was demonstrated by reduction of CEACAM1 levels at fasting and in Wy14,643-treated mice and FAO hepatoma cells. The relevance of this observation may relate to the role of CEACAM1 in regulating de novo lipogenesis in response to transient insulin surges at 4 and 7-8 hours of refeeding (Najjar et al., 2005), which are associated with a parallel increase in CEACAM1 protein levels (Figure 16A).

At fasting, lipogenic enzymes are maintained at low levels in order to limit de novo synthesis of fatty acids. Upon refeeding, insulin surges at 1, 4 and 7 hours of refeeding (Najjar et al., 2005). The earliest insulin effect to recover is the suppression of VLDL secretion (at ~1 hour of refeeding) (Zammit and Moir, 1994). This is followed by the slow recovery of malonyl-CoA levels (~ beginning at about 4 h of refeeding), which proceeds slowly in order not to inhibit β-oxidation and gluconeogenesis until glycogen synthesis from glucose-6-phosphate is complete and glycogen storage is repleted (~ 6-8 hours of refeeding) (Roden and Shulman, 1999; Sugden et al., 2002). Null mutations of PPARα does not affect glycogen content at fed state and its depletion at fasting, but
significantly impaired synthesis and repletion of glycogen in the first few hours of refeeding (6 hours) (Sugden et al., 2002). This assigns an important role for PPARα-mediated fatty acid oxidation in maintaining normal insulin action with respect to glycogen storage.

Why would CEACAM1 levels be lower during fasting than refeeding? Our laboratory has recently shown that insulin surges inhibit FAS activity at 4 and 7 hours of refeeding via CEACAM1 phosphorylation by the insulin receptor and its binding to FAS (Najjar et al., 2005). The current studies reveal that CEACAM1 and FAS levels recover during that time, but with earlier onset and higher amplitude of CEACAM1 than FAS until about 8 hours of refeeding, at which point the CEACAM1: FAS ratio is progressively reversed with a relative increase in FAS levels (and activity), continuing gradually until 24 hours of refeeding. Because high CEACAM1: FAS ratio in the first 8 hours of refeeding. Thus, we hypothesize that the pulsatile increase in CEACAM1 levels together with low FAS levels in the first few hours of refeeding reduces FAS activity and leads to a slow recovery of malonyl-CoA levels, which begins at about 4 hours of refeeding and continues slowly in order not to inhibit β-oxidation before the glycogen store is replenished. In this manner, changes of CEACAM1 levels in a fasting-refeeding paradigm appears to be an adaptation process to the metabolic changes from a lipolytic to a glycolytic pathway during the transition period from fasting to refeeding.

PPARα and insulin have opposing effects on hepatic CEACAM1 expression. Previous work has shown that insulin upregulates the promoter activity of Ceacam1 (Najjar et al., 1996), suggesting a positive role of this hormone on hepatic CEACAM1
levels. This is consistent with the predominant expression of CEACAM1 in liver, which is exposed to a 2- to 3-fold higher level of insulin in the portal than the systemic circulation (Ward et al., 1990). In contrast, we herein show that Wy14,643, a PPARα agonist, reduces hepatic CEACAM1 mRNA and protein levels significantly in fasted mice and in FAO rat hepatoma cells. This suggests that PPARα activation negatively regulates CEACAM1 levels when insulin levels are low. Additionally, feeding mice with HF diet for 30 days reduces hepatic Ceacam1 mRNA levels by ~60% in wild type PPARα+/+, but not PPARα−/− null mice (Figure 14). Moreover, when fed a HF diet, BL6 PPARα+/+, but not PPARα−/− mice developed visceral obesity and hyperinsulinemia despite maintaining normal insulin secretion (Table V). Thus, our data are in agreement with others demonstrating that null mutation of PPARα protects against diet-induced insulin resistance and hyperinsulinemia in mice backcrossed on the BL6 (Bernal-Mizrachi et al., 2003; Guerre-Millo et al., 2001). With fatty acid oxidation being deficient in PPARα−/− mice, these mice develop lipogenesis and increased fat content at fasting and when fed a HF diet (Guerre-Millo et al., 2001). Thus, in PPARα−/− mice, it appears that there is dissociation between altered fat metabolism and insulin action by a mechanism that is not yet understood. Our data indicate that BL6 mice are susceptible to dietary-dependent reduction in hepatic CEACAM1 levels and insulin resistance, consistent with the long standing observation that BL6 mice are more prone to diet-induced diabetes than other strains (Surwit et al., 1988). Moreover, we have shown that the effect of HF diet on CEACAM1 is mediated by PPARα activation in light of the fact that HF diet decreased CEACAM1 in PPARα+/+ but not PPARα−/− mice on the BL6 background. This
observation is supported by low expression of CEACAM1 at fasting (Figure 16), when PPARα is activated, and the marked decrease in CEACAM1 levels when mice and cells were treated with the PPARα specific agonist, Wy14,643 (Figures 12 and 15).
We hypothesize that high-fat feeding induces visceral obesity and increases FFA uptake in liver. The FFA in liver activates PPARα, which, in turn, reduces hepatic CEACAM1 levels. Reduction of hepatic CEACAM1 impairs insulin clearance and causes insulin resistance without frank diabetes. Therefore, reduction of hepatic CEACAM1 levels is an early event in the pathogenesis of diet-induced insulin resistance.

**Elevated FFA Uptake in Liver**

↓

**Activation of PPAR alpha**

↓

**Reduced Hepatic CEACAM1**

↓

**Impaired Insulin Clearance**

↓

**Insulin Resistance**
We herein present evidence that diet-induced obesity causes insulin resistance through reduction of hepatic CEACAM1, and that this constitutes an early mechanism in the pathogenesis of insulin resistance. This mechanism involves CEACAM1, PPARα and insulin signaling and their role in the development of insulin resistance which explains why PPARα null mice do not develop insulin resistance when fed a HF diet. With CEACAM1 being a cell surface protein, it could be targeted easier than intracellular proteins and this may uncover a mechanism by which therapeutic measures could be developed to prevent progression from insulin resistance to frank diabetes. Therefore, investigating the CEACAM1-dependent mechanisms by which diet-induced obesity causes insulin resistance is imperative to our understanding of the pathogenesis of obesity and T2DM, and to identifying molecular targets for the prevention and/or treatment of these diseases.
CONCLUSIONS

**Hypothesis 1.** High-fat intake induces visceral obesity and decreases hepatic CEACAM1 via a PPARα-dependent pathway.

In order to investigate the relationship between altered fat metabolism in visceral obesity and hepatic CEACAM1 levels, we screened several obese rat models for CEACAM content in liver including the fa/fa, ZDF, and Koletsky rats. All of these rats exhibit accumulation of white visceral fat along with elevation in plasma FFA and triglyceride levels, in addition to impairment of insulin clearance. Our data reveal reduced hepatic CEACAM1 content in liver of these obese rats. This finding led to a positive correlation between visceral obesity and decreased hepatic CEACAM levels.

Next, we fed WT mice a HF diet that provides 45% of calories from fat for 9-30 days in order to induce visceral obesity and increase plasma FFA levels. We observed that HF diet caused visceral obesity with an increasingly more effect after 30 days of feeding. We demonstrated that HF intake for 30 days is associated with reduction in hepatic CEACAM1 levels.

What is known about FFA is that they are natural ligands of PPARα, which modulate gene expression of proteins involved in fatty acid oxidation. This has led us to test the hypothesis that HF decreases CEACAM1 via FFA-mediated PPARα activation in liver. Our data show that 30 days of HF diet increased PPARα mRNA and protein levels and decreased hepatic CEACAM1 mRNA and proteins.
In support of the hypothesis that PPARα represses CEACAM1, CEACAM1 levels were decreased at fasting, which constitutes a physiological state during which PPARα is activated, and following activating PPARα by Wy14,643 both in cells and mice. The hypothesis was further supported by the observation that null mutation of Ppara protected against diet-induced insulin resistance and decrease in CEACAM1 levels.

**Hypothesis 2.** Reduction of hepatic CEACAM1 levels is an early event in the pathogenesis of diet-induced insulin resistance.

We tested this hypothesis by conducting a time course analysis in which we fed WT mice a high-fat diet for 9-30 days and measured insulin resistance and CEACAM1 levels. Our data demonstrated that HF intake caused a progressive decrease in hepatic CEACAM1 levels in parallel to its progressive effect on visceral obesity and plasma FFA, reaching a decrease by $\geq 60\%$ of CEACAM1 levels after 21-30 days, and that hepatic insulin resistance and glucose intolerance developed concomitantly to Ceacam1 downregulation. Our data also revealed that insulin receptor phosphorylation was altered in liver (Figure 8A) prior to WAT and muscle (Figure 8C). This suggests that HF diet-induced visceral obesity reduces CEACAM1 levels and subsequently, causes insulin resistance in liver.

In conclusion, our data assign a primary role for reduced hepatic CEACAM1 levels in the early development of diet-induced insulin resistance.
SUMMARY

High fat intake leads to visceral adiposity, elevation in plasma FFA levels, hepatic insulin resistance and impairment of insulin clearance. Elevation in plasma FFA from the developed visceral adipose tissue in turn, downregulates insulin action by modifying multiple signaling pathways. Impairment of insulin clearance and action is caused by reduction of hepatic CEACAM1 levels. This, in turn, causes hyperinsulinemia, secondary insulin resistance and altered fat metabolism. However, it is not known whether FFA can directly reduce CEACAM1 levels or through their interaction with PPARs or some other transcription factors or mediators which are important in lipid metabolism and regulation.

We have herein shown that HF diet (45% of calories from fat) for 30 days reduced CEACAM1 mRNA and protein levels by more than 50-60% (Figure 7). This occurs in the presence of higher plasma FFA, but not triglycerides levels (Table II). We hypothesized that high circulating FFA target the liver and decrease hepatic CEACAM1 content, which in turn, causes insulin resistance. In response to hepatic insulin resistance the pancreas responds by secreting more insulin to overcome the resistance, which leads to further hyperinsulinemia and hyperproliferation of the adipose tissue.

Moreover, our data draw a positive correlation between visceral obesity and reduction in hepatic CEACAM1 levels, which causes impairment of insulin clearance and altered lipid metabolism and insulin resistance.

We have also found that FFA reduce CEACAM1 levels by activating PPARα and before adipokines are released from the white adipose tissue.
Moreover, we observed that CEACAM1 levels are regulated by a fasting-refeeding paradigm. According to this model, when PPARα levels are highest at fasting, CEACAM1 levels are low and peak at 4 and 7-8 h of refeeding in parallel to insulin pulses (Najjar et al., 2005). Because of the role of CEACAM1 in the downregulatory effect of insulin on FAS activity during the first few hours of refeeding, we hypothesize that changes of CEACAM1 relative to FAS levels during this time are important in the repletion of glycogen stores in the first few hours of refeeding following an overnight fast. Thus, our studies show that regulation of CEACAM1 levels plays an important role in regulating fatty acid oxidation in the transition from a fasting to refeeding state.
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ABSTRACT

Insulin resistance is the hallmark of type 2 diabetes. CEACAM1, a substrate of insulin receptor in liver, regulates insulin action by promoting insulin clearance. Inactivation of CEACAM1 impairs insulin clearance and causes hyperinsulinemia, insulin resistance, dyslipidemia and visceral adiposity in transgenic mice. Moreover, CEACAM1 levels are significantly reduced in spontaneously obese rats. Thus, there is a strong association between visceral obesity, insulin resistance and reduced hepatic CEACAM1 level.

Free fatty acids (FFA) that are released from adipose tissue, in particular during fasting and obesity, are transported to liver to activate the transcription factor peroxisome proliferator-activated receptor α (PPARα), which regulates expression of genes involved in fatty acid transport and oxidation. Thus, we investigated whether PPARα downregulates CEACAM1 level and hence mediates insulin resistance in obese mice.

We fed normal wild-type male mice with a high-fat diet (HF) (45% of calories from fat), for 9 to 30 d. HF treatment for 9 d caused a loss of hepatic CEACAM1 mRNA and protein content by up to 35% without affecting insulin clearance and insulin action. However, treatment for 21-30 d reduced CEACAM1 mRNA and protein levels by ~≥ and led to impaired insulin clearance and insulin resistance in a reversible manner. On the other hand when we inhibited lipolysis in HF-fed mice by nicotinic acid, we observed normal hepatic CEACAM1 level and insulin action. Increased mobilization of
FFA from the adipose tissue, especially during fasting, increases FFA uptake by liver and their conversion into long chain fatty acids (LCFA) and LCFA-CoA. These activate the peroxisome proliferator-activated receptor α (PPARα, a nuclear transcription factor that upregulates transcription of proteins involved in fatty acid transport into mitochondria and oxidation in order to support gluconeogenesis). We have observed that at fasting, when PPARα levels are highest the level of CEACAM1 is low. Moreover, Wy14,643, a PPARα agonist, reduced hepatic CEACAM1 mRNA and protein levels significantly in mice and rat hepatoma cells (FAO). Additionally, feeding PPARα −/− mice HF 30 days reduced hepatic Ceacam1 mRNA levels by ~ 60% in wild-type PPARα +/+, but not PPARα −/− null mice, and this was associated with hyperinsulinemia and insulin resistance. Taken together, our data suggests that PPARα activation (by fasting, HF diet or Wy treatment) decreases hepatic CEACAM1 levels. In addition, our data suggest that reduction in hepatic CEACAM1 via PPARα-dependent pathway constitutes an early mechanism of diet-induced insulin resistance.