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entitled

A Role for CEACAM2 protein in Insulin Secretion, Clearance and Action

by

Simona S. Ghanem

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

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An Abstract of

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The carcinoembryonic antigen related cell adhesion molecule (CEACAM) family is a group of proteins reported to have potent roles in insulin signaling, lipogenesis, immunity, and tumor suppression. To investigate the role of CEACAM2, we have used a murine model with global Ceacam2 gene deletion. The male Ceacam2 knockout mice showed an increase in insulin release suggesting that CEACAM2 plays an important role in regulating insulin secretion. Due to the low expression of Ceacam2 in beta pancreatic cells, CEACAM2 effect on insulin secretion was caused by an extra-pancreatic mechanism. Consistent to the expression of CEACAM2 in the distal intestinal villi, null CEACAM2 male mice showed a higher excursion of GLP-1 in response to oral administration of glucose, showing that the CEACAM2 effect on insulin secretion is mediated partly via a GLP-1 dependent mechanism. In accordance with the importance of insulin clearance in the kidney to maintain normal plasma insulin levels and CEACAM2 expression in proximal tubule cells, mutant male mice revealed an impairment in renal insulin clearance reflecting its function in regulating insulin extraction in the kidney. These data also imply that CEACAM2 is an insulin receptor substrate that undergoes
phosphorylation at its intra cellular phosphorylation sites during insulin stimulation to mediate receptor-mediated endocytosis in proximal tubule cells. Despite the hyperphagia of all age groups, Ceacam2 knockout male mice are insulin sensitive till the age of 9 months. We also show that insulin resistance, obesity and hyperinsulinemia did not start until 9 months of age likely due to reduced energy expenditure and increased free fatty acids. In addition, CEACAM1 levels are reduced during that age group explaining the insulin resistance and reflecting the low insulin clearance (low C/I molar ratio). This illustrates that insulin resistance in these mutant mice is age dependent. These reports expand our understanding of the role of CEACAM2 protein in the regulation of insulin secretion and insulin action.
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Chapter 1

Literature Review

Metabolic syndrome is characterized by insulin resistance and considered a leading cause of mortality and morbidity. It consists of a group of metabolic abnormalities that increases the risk of health problems, such as type 2 diabetes (T2D) and cardiovascular disease (1, 2). These risk factors include visceral abdominal obesity, dyslipidemia and hypertension (2). According to growing epidemiological evidence, visceral or central obesity has been reported to be increasing worldwide with most Americans being overweight and almost 35% being obese (3).

Insulin and Its Secretion

Insulin is a peptide hormone that is synthesized and secreted from the beta pancreatic cells with a primary role in absorption and clearance of glucose from the bloodstream to maintain plasma glucose within a physiological range (4, 5). It also has additional functions including regulation of various cellular processes such as cell growth and proliferation making insulin an anabolic hormone. Glucose uptake and metabolism play an essential role in the control of insulin production. Moreover, it also has been reported as a strong transcriptional activator for the insulin
gene (6), that is found on the human chromosome 11p15.5 (7). The insulin gene undergoes a heavy transcriptional regulation (8). Several defects in insulin gene transcription have been indicated to cause various types of diabetes, especially the Mature-Onset Diabetes of the Youth (MODY) family of diseases (9). These diseases are caused by mutations in genes that encode transcription factors that relate to insulin production or glucose metabolism in the pancreas and eventually promote insulin secretion (10).

Insulin is initially transcribed into preproinsulin mRNA. After translation of the latter, preproinsulin polypeptide will then be translocated to the rough endoplasmic reticulum via a signaling sequence (11). This is followed by a proteolytic cleavage to form proinsulin polypeptide (12). Proinsulin is made of A and B chains that are linked by the connecting peptide (C-peptide) (13). Before being packaged into clathrin-coated granules, proinsulin folds to form two disulfide bridges between the A and B chains (14). Proinsulin molecules will then undergo cleavage to form C-peptide and functional insulin (15, 16). Mature active insulin proteins that are 5.8 kDa and C-peptide are stored in beta cell vesicles until they are exocytosed upon nutritional stimulation at a molar ratio of 1 (15).

The β endocrine cells of the pancreas are known to be the glucose-sensing cells that respond to glucose by secreting insulin (17, 18). Glucose enters the beta cell through specialized glucose transporters (GLUT-2) that will eventually undergo glycolysis to yield ATP (19). The ATP dependent channel (KATP) is a main component in beta cells comprising of the subunits SUR1 and Kir6.2 (18, 20-22). At low glucose concentrations and cellular ATP, potassium ion (K⁺) efflux through the open KATP channel in
pancreatic beta cells results in the closure of the voltage-gated calcium (Ca\(^{2+}\)) channels. On the other hand, an increase in glucose uptake and a subsequent rise in cytosolic nucleotide concentration results in the closure and inactivation of KATP channels (23) creating a state of membrane depolarization that will open L-type voltage dependent Ca\(^{2+}\) channels, and cause Ca\(^{2+}\) influx and, finally, exocytosis of insulin vesicles (18, 24-26). Secreted insulin also promotes more insulin synthesis by pancreatic activation of insulin and IGF receptors in the beta cells (27). This provides evidence of the link between cellular metabolism and the process of insulin secretion through these ATP sensitive potassium channels (28).

**Insulin Receptor**

After insulin secretion, this hormone acts on its specific plasma membrane receptor, the insulin receptor (IR), leading to a decrease in plasma glucose levels to reach its normal range. Binding of insulin to its receptor starts a cascade of signaling events that regulate various processes including glucose transport, cell growth, synthesis of glycogen, proteins and fatty acids and eventually mediate insulin action. Insulin mostly acts on its receptor in the insulin target tissues, where the latter is highly expressed in liver, brain, muscle and adipose tissue. The insulin receptor is made of two α and two β subunits, linked by disulfide bridges (29), and that phosphorylate upon insulin binding, making this receptor a member of the receptor tyrosine kinase family. The α subunit is extracellular and contains the insulin-binding region, while the β subunit is composed of an extracellular domain, a single transmembrane helix and the cytosolic tyrosine kinase domain (30). Two molecules of insulin are able to bind to the IR dimer (4). Upon binding
of insulin to its receptor, all tyrosine residues undergo autophosphorylation, which ultimately allows the phosphorylation of insulin receptor substrate (IRS) proteins (31). Following that, IRS proteins phosphorylate the Src homology 2 (SH2) domains of other proteins, such as phosphatidylinositol 3 kinase (PI3-kinase), to stimulate downstream pathways (32).

**Insulin Action in Muscle and Adipose Tissue**

The predominant form of glucose transporter in muscle and adipose tissue is the glucose transporter 4 (GLUT4). These two tissues play important roles in glucose disposal, the muscle being the major site of glucose uptake (33). Insulin acts on these target tissues by binding to the insulin receptor (IR) leading to its phosphorylation thus activating IRS1 or IRS2, followed by PI3K and AKT phosphorylation (33). This process stimulates the translocation of GLUT4 from the storage vesicles to the surface membrane allowing glucose influx in the cell (34). During fasting conditions and when insulin levels decrease, GLUT4 shifts its presence from the cell membrane back to the storage vesicle waiting for later insulin signaling.

**Insulin Action in the Liver**

Insulin signaling in the liver is essential to maintain glucose homeostasis of the body by inhibiting gluconeogenesis and stimulating glycogen synthesis. The liver expresses the glucose transporter 2 (GLUT2) that is considered insulin independent; its translocation to the cell surface does not require insulin action. Upon insulin signaling in the hepatocytes, the forkhead transcription factor protein FOXO1 is phosphorylated through the Akt
pathway making it leave the nucleus to the cytoplasm. This process will decrease transcription of gluconeogenic genes and hepatic glucose formation (35). During low glucose levels, FOXO1 residues that are unphosphorylated stay in the nucleus that will eventually promote the transcription of the gluconeogenic genes like glucose-6-phosphatase and Pepck (36).

**Glucagon Like Peptide 1**

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted from the intestinal endocrine L cells in response to nutrient ingestion, the primary physiological stimulus of L cells (37, 38). The L cells are a subset of the intestinal cells that are found mostly in the distal epithelium part of the small intestine most likely in ileum and colon (17). GLP-1 regulates glycemia levels by stimulating glucose-stimulated insulin secretion from the pancreatic beta cells (39, 40). Similarly to the α and β pancreatic cells, the intestinal endocrine L cells are also considered to be glucose sensing cells that secrete glucagon like peptide 1, GLP-1, in response to glucose (17, 41). These cells share the same machinery as the pancreatic cells, expressing KATP channels consisting of the common subunits, SUR1 and Kir6.2 (17). GLUTag cells, immortalized intestinal L-cells, are the most reliable cell line that can be used to study the mechanism of GLP-1 secretion (42, 43).
**ATP dependent Potassium Channels**

The adenosine 5’ triphosphate-sensitive potassium (KATP) channel is considered an octameric complex of 4 Kir6.x and 4 SURx subunits (23, 44-48). The pore of this channel consists of four identical Kir6.x subunits each one of which is associated with one regulatory subunit, SUR (49, 50). The Kir6.x subunit has two transmembrane domains and forms the channel pore; it also belongs to the inwardly rectifying family of potassium channels (21, 22, 51). The N terminus of the first transmembrane domain of KIR6.X is reported to interact with the SUR subunit (52). This subunit can exist in either one of its two isoforms: Kir6.1 and Kir6.2 (22, 51). The sulfonylurea receptor (SUR) belongs to the ATP-binding cassette (ABC) transporter family and has 17 transmembrane domains with one group made of 5 helices and the following two groups made of 6 helices, each of which are followed by a large cytosolic loop containing important nucleotide binding sites (49, 53, 54). It acts as a nucleotide mediated regulatory subunit since it has two nucleotide binding domains on the cytoplasmic side, and there are two isoforms: SUR1 and SUR2 (46-48). No potassium channel activity was reported when either SUR or Kir subunit was expressed alone. Moreover, SUR1 and Kir6.2 genes are located on chromosome 11p15.1 (55).

KATP channels function as metabolic sensors that couple the cellular metabolism to electrical activity of the plasma membrane by regulating flux of potassium ions (K+) (56-58). Decrease in metabolism activates KATP channels leading to its opening and eventually K+ efflux, followed by membrane hyperpolarization (59, 60). On the other hand, increased metabolism causes an increase in ATP that closes the channels and
inhibits its activity, resulting in membrane depolarization and subsequent cellular responses (59, 61-63).

KATP channels are expressed in several tissues, such as the skeletal and smooth muscle, heart, kidney, pancreatic β cells, pituitary, brain, and others (64-66). These channels play various physiological roles including regulating insulin secretion from pancreatic β cells (66-71), glucagon secretion from pancreatic α cells (72), somatostatin secretion from D cells (73), food intake (74), K⁺ efflux in response to Na⁺ reabsorption (75), and GLP-1 secretion from L cells (42). Moreover, ventromedial hypothalamic neurons known as glucose-responsive neurons, are important in glucose sensing and mediate the counter-regulatory response to glucose (20, 76). Arcuate nucleus neurons are also involved in appetite regulation (77).

KATP channels are regulated by certain cytosolic factors, specifically adenine nucleotides such as ATP and ADP. These nucleotides interact with two sites on the channel, one inhibitory and the other stimulatory respectively (78-81). ATP inhibits the channels’ activity whereas ADP in the presence of Mg²⁺ reverses this inhibitory effect causing the channel’s stimulation and activation (78, 81, 82). In normal physiological cellular states where Mg²⁺ is always present, channel activity is determined by the ATP/ADP ratio or balance between the inhibitory and stimulatory effects of nucleotides. The increase in the ATP/ADP ratio inhibits the KATP channel while the decrease activates it (83). Blockage and closure of the channel results from the binding of ATP to the intracellular domains of Kir6.x (80, 84-86), whereas this is reversed by stimulation of the channel via the interaction of SURx subunit and MgADP, through its two cytosolic nucleotide-binding domains (NBD) (87-89). It is believed that the binding of nucleotides
to SUR regulates the activity of the channel via inducing a conformational change of the channel through the interactions between SUR and Kir subunits.

Appropriate trafficking of KATP subunits to the cell surface dictates functional channels. This is controlled by a tri-peptide endoplasmic reticulum (ER)-retention signal, RKR, that is found in both SUR1 and Kir6.2 subunits. Exposure of this signal causes the subunits to be trapped in the endoplasmic reticulum, whereas, under normal conditions and correct stoichiometry both subunits interact together to mask RKR allowing the channel to facilitate cellular translocation and its movement to the cell surface (82).

Other than adenine nucleotides, SUR subunits have been reported to be sensitive to sulfonylureas or channel blockers such as tolbutamide and glibenclamide (24, 55) and channel openers such as diazoxide (90, 91), where the former agents are used to treat type 2 diabetes since they inhibit KATP channels to stimulate insulin secretion from pancreatic cells (53, 68).

Many diseases have been associated with mutations or deletions of the KATP channels, whereby the loss of these ATP dependent channels have an effect on causing a persistent calcium influx leading to increased insulin secretion and eventually hyperinsulinism causing familial hypoglycemia (68).

CEACAM1

The Carcino-Antigen Related Cell Adhesion Molecule 1 (CEACAM1) protein is a highly conserved transmembrane glycoprotein and member of the immunoglobulin (IgG) gene family having an extracellular region containing up to four immunoglobulin loops, and an
intracellular region containing serine and tyrosine phosphorylation sites (92). CEACAM1 was also referred to as biliary glycoprotein 1 (Bgp1) encoded by a single gene nearly 17 kilobases in length and composed of a functional promoter with 9 exons and 8 introns found on chromosome 7 (92, 93). Concerning its tissue distribution, Ceacam1 is fairly ubiquitously expressed, in the pancreas, kidney, endometrium, epithelial cells of the intestine, hematopoietic cells, and predominantly in the liver (94, 95). CEACAM1 protein appears to be in two forms: the long and the short isoform; alternative splicing of Ceacam1 transcript causes the deletion of the seventh exon, resulting in the short isoform. This short isoform (CEACAM1-4S) lacks 61 residues of the 71 amino acids of the intracellular domain that characterize the long isoform (CEACAM1-4L), in addition to the loss of the essential intracellular phosphorylation sites (Ser503) and tyrosine (Tyr488) (92, 96). Many studies have reported CEACAM1 functions including cell adhesion (97), hepatic insulin clearance (98-100), angiogenesis (101, 102), tumor suppression (103-106), and anti-inflammation (107).

CEACAM1 was originally identified as a substrate of the insulin receptor (IR) (108) and the epidermal growth factor receptor (109). Phosphorylation of CEACAM1, mediated by insulin receptor tyrosine kinase occurs in hepatocytes of the liver, but not in muscle or adipose tissues (110). Upon insulin stimulation, phosphorylation of Tyr1316 of the IR indirectly phosphorylates Tyr488 on the intracellular domain of CEACAM1 (111). This should be accompanied by the activation (phosphorylation) of Ser503 site on CEACAM1 (110). Previous studies have shown that inactivation of either the residues Y488 or S503 completely inhibit the phosphorylation of CEACAM1 by the insulin receptor, on the other hand, mutation of Y513 did not affect the phosphorylation (110). Following
CEACAM1 phosphorylation, Tyr488 on CEACAM1 binds to the SH2 domain of Shc, another known substrate of IR (112) to form the complex IR-CEACAM1-Shc allowing endocytosis via clathrin coated vesicles (113, 114) and degradation of insulin (115) in the lysosome followed by recycling of IR to the plasma membrane (116). In cases of inactivation of CEACAM1 in mice, disruption of this IR-CEACAM1-Shc complex internalization will occur leading to impairment of insulin clearance (112). Contrary to this, Y513 residue is important for dephosphorylation of CEACAM1 (117, 118). As previously reported, SHP-1 and SHP-2 phosphatases dephosphorylate CEACAM1 (119).

Mice with impaired insulin clearance have been reported to show insulin resistance in the liver and muscle, obesity, elevated free fatty acids (FFA) and triglycerides (TG) (98, 120). In addition to mice, this phenotype of metabolic abnormalities has been shown to be present in rats associated with decreased levels of CEACAM1 (121).

Important evidence supported the hypothesis of the regulatory function of CEACAM1 on insulin action by promoting insulin clearance through the L-SACC1 mouse with liver-specific overexpression of the dominant negative phosphorylation-defective S503A CEACAM1 mutant. This mouse model developed hyperinsulinemia resulting from impaired insulin clearance (98). Hyperinsulinemia led to insulin resistance (120). Moreover, this L-SACC1 mouse also showed visceral adiposity with elevated plasma FFA and plasma and hepatic triglyceride levels. In addition to L-SACC1 mice, the global knockout of Ceacam1 (Cc1^-/^-) presented impairment of insulin clearance, hyperinsulinemia, elevated serum and hepatic triglyceride levels, and an increase in body mass (99) to further emphasize the primary role of CEACAM1 in promoting insulin clearance in the liver (98, 122).
CEACAM1 also exerts a major role in regulating de novo lipogenesis in the liver by mediating the negative acute effect of insulin on fatty acid synthase (FAS) enzymatic activity. FAS is a crucial enzyme in the de novo synthesis of fatty acids. This enzyme converts malonyl-CoA to palmitate, a well-known saturated fatty acid. Malonyl-CoA will dictate carnitine palmitoyltransferase 1 (CPT-1) levels by its allosteric inhibition, the later being a main enzyme in promoting fatty acid oxidation. During normal levels of insulin, a decrease in FAS activity occurs, mediated by an acute rise of insulin to induce CEACAM1 phosphorylation and internalization as part of insulin endocytosis and binding to FAS (123) contributing to hepatic insulin sensitivity. During chronic insulin levels, as in cases of hyperinsulinemia and insulin resistance, upon which insulin pulsatility is absent and phosphorylated CEACAM1 levels are reduced, induction of lipogenesis causes an increase in the transcription of Fasn (gene encoding for FAS) and other lipogenic genes (124, 125) to induce de novo hepatic lipogenesis.

**CEACAM2**

Just as Ceacam1 has been widely studied, its twin and related gene, the Carcino-Antigen Related Cell Adhesion Molecule 2 (CEACAM2), is also highly investigated with many reports targeting his various functions. The gene Ceacam2 was shown to be on chromosome 7 first in BALB/c and C57BL/6 mice and referred as biliary glycoprotein 2 (Bgp2) at the beginning to be further identified as Ceacam2 (126). Ceacam2 was reported to be 25 kilobases long (93). In contrast to CEACAM1, CEACAM2 does not work as a cell adhesion molecule due to divergent amino acid sequences on the extracellular tail even though they share high homology of gene sequences that contain nine exons and
eight introns (127). In both genes, the seventh exon can undergo alternative splicing that will form distinct transcripts yielding either the long isoform (\(-L\)) or the short isoform (\(-S\)) that lacks the conserved phosphorylation sites (92, 127). Moreover, CEACAM2 can also undergo alternative splicing at exons 3 and 4 forming a shorter extra cellular domain containing only two out of four immunoglobulin loops. Therefore, CEACAM2 exists mostly as CEACAM2L/2S.

The transmembrane glycoprotein, CEACAM2, has a distinct tissue distribution that gives it unique functions. Ceacam2 expression has been identified in the kidney, spleen, testis, prostate, platelets, crypt epithelial cells and brain including the ventromedial hypothalamus (VMH) (93, 128-131). This protein is not found in the peripheral insulin target tissues such as the liver, muscle and white adipose tissue. Our mouse model of global deletion of CEACAM2 was the first to report CEACAM2 important function in regulation of energy balance (including regulation of food intake, energy expenditure and brown adipogenesis) (128, 132). Recent reports have also expanded CEACAM2 functions by showing its importance in platelet activation/adhesion and thrombus stability (131), spermatid maturation (130) and insulin secretion (133). Consistent with CEACAM2 expression in the VMH, Ceacam2 null deletion (\(Cc^{2/}\)) showed hyperphagia in both males and females. Despite the increase in food intake, only females showed insulin resistance, hyperinsulinemia, and obesity; this was not evident in males due to the increase in energy expenditure partly mediated by the increase in brown adipogenesis and activated sympathetic nervous outflow to the adipose tissue (128, 132). Moreover, global knockout of CEACAM2 caused an increase in insulin secretion in males, implicating
CEACAM2 main role in the regulation of insulin secretion (133). This has been shown to be partly mediated via a glucagon-like peptide (GLP-1) mediated mechanism.

Other than tissue distribution, metabolic state also appears to regulate CEACAM1 and CEACAM2 proteins expression, whereby CEACAM2 is expressed during the fasting state and to a lower extent when glucose levels are high (128). On the other hand, CEACAM1 is more highly expressed in the fed state when insulin levels increase compared to the fasting state.

Insulin action depends on the amount of circulating insulin in the blood circulation. The level of plasma insulin is regulated by two major processes, insulin secretion from pancreatic beta cells and insulin clearance occurring mainly in the liver and to a minor extent in the kidney. The observation of increased plasma insulin levels after oral glucose administration and lower renal insulin clearance in Cc2 / male mice provided evidence that CEACAM2 has an important role in maintaining insulin sensitivity by regulating insulin secretion and promoting renal insulin clearance. To this end, we fully characterized the metabolic phenotype of male mice and elucidated the mechanisms underlying the role of CEACAM2 in insulin secretion, renal clearance and action.
Chapter 2

Increased Glucose-induced Secretion of Glucagon-like Peptide-1 in Mice Lacking the Carcinoembryonic Antigen-related Cell Adhesion Molecule 2 (CEACAM2)


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Abstract

Carcinoembryonic antigen-related cell adhesion molecule 2 (CEACAM2) regulates food intake as demonstrated by hyperphagia in mice with the Ceacam2 null mutation (Cc2−/−). This study investigated whether CEACAM2 also regulates insulin secretion. Ceacam2 deletion caused an increase in b-cell secretory function, as assessed by hyperglycemic clamp analysis, without affecting insulin response. Although CEACAM2 is expressed in pancreatic islets predominantly in non-b-cells, basal plasma levels of insulin, glucagon and somatostatin, islet areas, and glucose-induced insulin secretion in pooled Cc2−/− islets were all normal. Consistent with immunofluorescence analysis showing CEACAM2 expression in distal intestinal villi, Cc2−/− mice exhibited a higher release of oral glucose-mediated GLP-1, an incretin that potentiates insulin secretion in response to glucose. Compared with wild type, Cc2−/− mice also showed a higher insulin excursion during the oral glucose tolerance test. Pretreating with exendin (9–39), a GLP-1 receptor antagonist, suppressed the effect of Ceacam2 deletion on glucose-induced insulin secretion. Moreover, GLP-1 release into the medium of GLUTag enteroendocrine cells was increased with siRNA-mediated Ceacam2 down-regulation in parallel to an increase in Ca2+ entry through L-type voltage-dependent Ca2+ channels. Thus, CEACAM2 regulates insulin secretion, at least in part, by a GLP-1-mediated mechanism, independent of confounding metabolic factors.
Introduction

CEACAM2 (carcinoembryonic antigen-related cell adhesion molecule 2) is a transmembrane glycoprotein that is expressed in kidney, spleen, testis, platelets, crypt epithelial cells of the small intestine, and in some brain nuclei, including the ventromedial hypothalamus (Heinrich et al., 2010, Salaheldeen et al., 2012, Alshahrani et al., 2014, Han et al., 2001, Robitaille et al., 1999). Consistently, CEACAM2 is implicated in the central regulation of energy balance (including regulation of food intake, energy expenditure, and brown adipogenesis) (Heinrich et al., 2010, Patel et al., 2012), platelet activation/adhesion and thrombus stability (Alshahrani et al., 2014), and spermatid maturation (Salaheldeen et al., 2012).

CEACAM2 is highly homologous to CEACAM1, the protein product of a distinct gene. Both genes contain nine exons, the seventh of which undergoes alternative splicing to yield different transcripts that are distinguished by a long (-L) or a short (-S) intracellular tail, containing or devoid of conserved phosphorylation sites (Robitaille et al., 1999, Najjar et al., 1993). In particular, in the case of CEACAM2, exons 3 and 4 commonly undergo alternative splicing to yield a shorter extracellular domain containing two instead of four immunoglobulin-like loops. Hence, CEACAM2 is mostly expressed as CEACAM2–2L/2S.

Although CEACAM2 shares some functions with CEACAM1, such as platelet activation/adhesion (Alshahrani et al., 2014), it does not appear to directly act as a cell adhesion molecule (Robitaille et al., 1999). Moreover, the absence of its transcripts in hepatocytes has not warranted the investigation of its role in hepatic insulin clearance, as was the case for CEACAM1 (Xu et al., 2009, DeAngelis et al., 2008, Poy et al., 2002).
Mice with Ceacam2 null deletion (Cc2−/−) exhibited hyperphagia (Heinrich et al., 2010, Patel et al., 2012). Because of increased energy expenditure, hyperphagia did not translate into insulin resistance in males (Patel et al., 2012), as it did to females (Heinrich et al., 2010). This study investigated whether CEACAM2 regulates insulin secretion. We report here that CEACAM2 plays a role in insulin secretion via a mechanism implicating the release of the insulinotropic glucagon-like peptide-1 (GLP-1), an incretin that potentiates glucose-stimulated insulin secretion from pancreatic b-cells (D’Alessio et al., 1994, Shalev 1997, Reimann et al., 2002, Kedees et al., 2013, Habib et al., 2013, Seino et al., 2013, Drucker 2015).

Experimental Procedures

Mouse Generation—The generation of Cc2−/− mice was described previously (Heinrich et al., 2010, Patel et al., 2012). Unless otherwise mentioned, male mice (5–7 months of age) were used. All animals were housed in a 12-h dark-light cycle and fed standard chow (Harlan Teklad 2016; Harlan, Haslett, MI) ad libitum at the Division of Laboratory Animal Resources at the University of Toledo College of Medicine. All procedures and animal experiments were approved by the Institutional Animal Care and Utilization Committee.

Plasma Biochemistry—Mice were anesthetized with sodium pentobarbital at 11:00 a.m. following an overnight fast. Whole venous blood was drawn from the retro-orbital sinuses to measure, by radioimmunoassay (RIA), the plasma levels of insulin, C-peptide (Linco Research, Billerica, MA), somatostatin (Phoenix Pharmaceuticals, Belmont, CA),
and total GLP-1 (catalog no. GLP1T-36HK, Millipore, Billerica, MA). RIA (Linco) was used to measure basal plasma glucagon levels in mice fasted for 4 h (from 07:00 to 11:00).

**Glucose Tolerance Test**—Male mice (n=5/genotype, 5 months old) were fasted overnight from 17:00 to 08:00 the following day before being anesthetized and administered glucose via oral gavage (3.0 g/kg BWT). Blood was drawn from the retro-orbital sinus to measure blood glucose and plasma insulin levels at 0 –120 min post-glucose administration. In parallel experiments, anesthetized mice were injected intraperitoneally with 5 mg of the GLP-1 receptor antagonist, exendin (9–39) (American Peptide Co. Inc., Sunnyvale, CA), 20 min before glucose administration.

**Hyperglycemic Clamp Analysis in Awake Mice**—To assess b-cell function in vivo, a 2-h hyperglycemic clamp was performed as described previously (Cho et al., 2007). Male mice (n=7/genotype, 7 months of age) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg BWT) and xylazine (10 mg/kg BWT), and an indwelling catheter was inserted into the right internal jugular vein. Four-to-five days later, mice were fasted overnight before being subjected to hyperglycemic clamp analysis with a continuous infusion of 20% glucose to raise and maintain plasma glucose levels at 300 mg/dl for 2 h. Blood samples (40 ml) were collected at 15–20-min intervals over a period of 120 min to measure plasma glucose and insulin concentrations.

**Hyperinsulinemia-Euglycemic Clamp Analysis**—A 2-h hyperinsulinemic-euglycemic clamp was performed in overnight-fasted awake mice (n = 11/genotype, 7–8 months old) with primed and continuous infusion of human regular insulin (Humulin; Lilly) at a rate of 2.5 milliunits.kg⁻¹.min⁻¹, as described (Patel et al., 2012). Glucose metabolism was
estimated with a continuous infusion of [3-3H] glucose (PerkinElmer Life Sciences) for 2 h prior to (1850 Bq/min) and throughout the clamps (3700 Bq/min).

*Plasma GLP-1 Measurement in Response to Oral Glucose*— Mice (n ≥6/genotype) were fasted overnight, subjected to an oral glucose administration (3 g/kg BWT), and anesthetized with pentobarbital immediately after glucose administration before their retro-orbital blood was drawn 30 min later to measure blood glucose and plasma insulin levels. For GLP-1 measurement, plasma from each genotype was pooled to determine GLP-1 levels in triplicate in 300µl aliquots, as above.

*Immunohistochemical Analysis*—Six-month-old male mice were anesthetized with pentobarbital, and their pancreases carefully dissected, cleared of fat and spleen, weighed, and fixed overnight in 4% paraformaldehyde. Tissues were embedded in paraffin and consecutive 7µm sections were mounted on slides. Sections were then stained with antibodies against insulin (Dako, Carpinteria, CA), glucagon (Sigma), somatostatin (Chemicon, Temecula, CA), pancreatic polypeptide (PP) (Linco), or a mixture of glucagon, somatostatin, and polypeptide (3Ab) and visualized utilizing 3,3’-diaminobenzidine tetra-hydrochloride reaction, as described (Kitamura et al., 2009).

*Immunofluorescence Staining*—As described previously (DeAngelis et al., 2008), small intestinal tissues were fixed overnight in Bouin’s solution, embedded in paraffin, and cut into 4µm consecutive sections. Following deparaffinization, sections were exposed to antigen retrieval by carefully boiling in a microwave oven in 10 mM citrate buffer, pH 6.0. Sections were then incubated at 4°C overnight with α-CEACAM2 custom-made anti-peptide polyclonal antibody raised in rabbit against the keyhole limpet hemocyanin-conjugated HPLC-purified peptide CNAEIVR-FVTGTKTKIHKPVH in CEACAM2
Subsequently, sections were incubated with a goat α-rabbit Cy5 (final dilution 1:400, Dianova 111-175-144) for 1 h to visualize with epifluorescence (Keyence BZ9000) equipped with a Plan Apo objective (Nikon). DAPI (1:9000) was used to visualize nuclei.

**Fluorescence-activated Cell Sorter Purification of Isolated Islets**—Islets were isolated by the intraductal collagenase digestion method (Kulkarni et al., 1999). After PBS wash, the suspension was passed through a 35µm filter before fluorescence-activated cell sorter (FACS) analysis, based on autofluorescence and size (Josefsen et al., 1996). Cells were sorted directly into TRIzol reagent, and the purity of the sorted fractions was determined by real time PCR for insulin and glucagon in each fraction.

**Insulin Secretion from Isolated Islets**—Purification of islets was conducted on pancreases of 4-month-old mice by collagenase digestion (Kitamura et al., 2001). Islets were then resuspended in RPMI 1640 medium containing 10% newborn calf serum and 5.5 mM glucose and cultured overnight at 37 °C. Islets were stimulated with glucose (2.8 –16.8 mM) or 20 mM KCl for 1 h at 37 °C, followed by centrifugation to assay insulin content by radioimmunoassay in the supernatant. Islets were dissolved in high salt buffer and sonicated three times at 80 watts for 10 s to measure DNA concentration for normalization.

**GLP-1 Release from Enteroendocrine Intestinal Cells**—GLUTag cells (generated by the D. Drucker laboratory at University of Toronto, Ontario, Canada) were maintained in 6-well plates in low glucose (5.6 mmol/liter) DMEM containing 10% fetal bovine serum and 1% penicillin and streptomycin at 37 °C in a 5% CO₂ incubator. Using Lipofectamine 2000 reagent (Invitrogen), cells were transfected with 100 pmol of
scrambled oligos or with a combination of 33.3 pmol each of Ceacam2-specific siRNA oligos (oligo 1, \texttt{caccactgcacaagtgactgttat}\textsuperscript{116} (exon2); oligo 2, \texttt{tgtaggttaaccagg}\textsuperscript{255} (exon2); oligo 3, \texttt{ggtctctctgtt}\textsuperscript{1345} (exon6)), based on the lowest shared homology between \textit{Ceacam1} and \textit{Ceacam2} in exons 2 and 6 (Han et al., 2001). Transfected cells were plated on Matrigel-coated 6-well culture plates. 2–3 days post-transfection, GLP-1 secretion was assessed as described previously (Parker et al., 2012). Briefly, cells were washed and incubated in saline solution containing in millimolar (128 NaCl, 5.6 KCl, 4.2 NaHCO\textsubscript{3}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 2.6 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, 10 HEPES, pH 7.4) and 1.6 mmol/liter glucose for 2 h at 37 °C. Some cells were treated with 11 mM glucose for an additional 2 h before the medium was removed and stored at 80 °C, and cells were lysed in 900 µl of RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Triton, 0.5% deoxycholic acid, and complete EDTA-free protease inhibitor mixture (Roche Applied Science). GLP-1 was measured in triplicate in 300 µl of medium and of cell lysates using the Millipore RIA kit, as above. Secreted GLP-1 was calculated as percent of GLP-1 in media relative to the sum of GLP-1 content in medium plus cell lysates (Iakoubov et al., 2007).

\textit{Live Cell Fluorescence} and \textit{Intracellular Ca\textsuperscript{2+} Measurements}—GLUTag cells were plated on Matrigel-coated coverslips (Corning Glass) 1 day prior to undergoing transfection with scrambled or Ceacam2-specific siRNA oligos, as above. Two to 3 days later, cells were loaded with 2 µM Fura-2 AM in physiological saline solution (147 mM NaCl, 5 mM KCl, 2.2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, and 1 mM D-glucose) for 1 h at room temperature and washed. Coverslips were mounted, and intracellular Ca\textsuperscript{2+} measurements were obtained using a Nikon TE2000 microscope.
Live cell imaging was performed using a Polychrome IV monochromator-based high speed digital imaging system (TILL Photonics, Gräfelfing, Germany) ported to a fiber optic guide and epifluorescence condenser and coupled to a Nikon TE2000 microscope. Cytosolic Ca$^{2+}$ concentration dynamics in Fura-2 AM-loaded GLUTag cells was recorded by alternately illuminating cells with 340 and 380 nm wavelength light focused onto the image plane via a DM400 dichroic mirror and Nikon Super-Fluor X40 oil immersion objective, and fluorescence was obtained through a 525 ± 25-nm bandpass filter (Chroma Technologies, Brattleboro, VT). Acquired images were analyzed using TILLvisION (TILL Photonics) and ImageJ (W. S. Rasband, National Institutes of Health, Bethesda).

**Western Blot Analysis**—Tissues were lysed in 150 mM NaCl, 50 mM HEPES, pH 7.6, containing protease and phosphatase inhibitor, and the protein concentration was determined by BCA protein assay (Pierce) prior to analysis by 7% SDS-PAGE and immunoprobing with specific antibodies. These include polyclonal antibodies against mouse CEACAM1 (α-mCC1) (Ab-231) (DeAngelis et al., 2008) and mouse CEACAM2 (α-mCC2) (above), in addition to monoclonal antibodies against tubulin (Sigma) and GAPDH (Santa Cruz Biotechnology, Inc., Dallas, TX) to normalize for the amount of proteins analyzed. Blots were incubated with horseradish peroxidase conjugated with anti-rabbit and anti-mouse IgG (Amersham Biosciences) antibodies, and proteins were detected by enhanced chemiluminescence (ECL; Amersham Biosciences).

**Semi-quantitative Real Time PCR Analysis**—RNA was extracted using the TRIzol method according to the manufacturer’s protocol. Following DNase digestion (DNasefree, Ambion), 200 ng of RNA was transcribed into cDNA in a 20 μl reaction using a High Capacity cDNA archive kit (Applied Biosystems), analyzed, and amplified (ABI7900 HT
system). PCR was performed in a 10 µl reaction, containing 5 µl of cDNA (1/5 diluted), 1 SYBR Green PCR Master Mix (Applied Biosystems) and 300 nM of each primer as follows: Ceacam2: F, 5'-CTACTGCTCACAGCCTCACTTTTAG-3', and R, 5'-GCTAAAGGCCAAGACTCCCTTTCAT-3'; Ceacam1: F, 5'-CTAC-TGCTCACAGCCTCACTTTTAG-3', and R, 5'-AAAGGCT-CCAAGCGCCAGGGG-3'; proglucagon: F, 5'-CAATGTTG-TTCCGGTTTCTC-3', and R, 5'-CCCTGATGAGATGAATGAAGACA-3'; 18S: F, 5'-TTCGAACGTCTGCCCTAT-CAA-3', and R, 5'-ATGGTAGGCACGGCGACTA-3'; and β-actin: F, 5'-AGGGCTATGCTCTCCCTCAC-3', and R, 5'-AAGGAAGGCTGGAAAAGAGC-3'. Ct values (cycle threshold) were used to calculate the amount of amplified PCR product relative to β-actin. The relative amount of mRNA was calculated as $2^{-\Delta Ct}$. Results were expressed in fold change as means ± S.E.

**Statistical Analysis**—Data were analyzed with SPSS software using one-way analysis of variance, two-way analysis of variance, or Student’s t test, as appropriate. For live cell imaging, Graph Pad Prism 3 (Graph Pad Software Inc., La Jolla) software packages were used. Values were represented as mean ± S.E., and $p < 0.05$ values were statistically significant.

**Results**

**Expression of CEACAM2 in Endocrine Pancreas**—Semi-quantitative RT-PCR analysis revealed that Ceacam2 mRNA is expressed at a ratio of 2:1 in FACS-purified mouse pancreatic non-β-cells relative to β-cells (Fig. 1A, panel i), as opposed to the Ceacam1
transcript, which was predominantly expressed in $\beta$-cells (Fig. 1A, panel ii), consistent with its protein distribution (DeAngelis et al., 2008).

Effect of CEACAM2 on Insulin Secretion—Five-month-old male $Cc2^{-/-}$ mice exhibited a 2-fold higher plasma C-peptide level than their $Cc2^{+/+}$ wild-type counterparts (Table 1). However, this did not translate into changes in plasma insulin levels, possibly because of the countervailing effect of increased insulin clearance, as measured by steady-state C-peptide/insulin molar ratio (Table 1), and consistent with maintenance of the protein level of hepatic CEACAM1 (Fig. 1B), a main promoter of insulin clearance in liver (Xu et al., 2009, DeAngelis et al., 2008, Poy et al., 2002), and its insulin-stimulated phosphorylation in the insulin-sensitive male mouse (Patel et al., 2012). Changes in plasma C-peptide levels are not likely attributed to a regulatory action by CEACAM1 on insulin secretion from pancreatic $b$-cells because it does not play a significant role in insulin secretion, as it does in insulin clearance (DeAngelis et al., 2008), and its cDNA level is preserved in pooled islets isolated from $Cc2^{-/-}$ mice, as RT-PCR amplification revealed (Fig. 1C).

Immunohistochemical analysis with anti-insulin, anti-glucagon, and anti-somatostatin antibodies, respectively (Fig. 2A), revealed normal areas of $\beta$-, $\alpha$-, and $\delta$-cells in 6-month-old $Cc2^{-/-}$ mice. Immunostaining with a three antibody mixture (3Ab) to glucagon, somatostatin, and pancreatic polypeptide P (PP) also revealed no difference in the area of cells secreting these hormones between the two groups of mice. Consistently, basal plasma levels of somatostatin and glucagon were normal in $Cc2^{-/-}$ as compared with $Cc^{+/+}$ mice (Table 1). Furthermore, glucose (2.8 –16.8 mM) and KCl (20 mM), a membrane-depolarizing agent, induced a comparable level of insulin release from pooled islets isolated from null or control mice (4-month-old) (Fig. 2B). Taken together, the data
suggest that Ceacam2 deletion from the pancreas does not intrinsically affect β-cell area or insulin secretion.

To further test insulin secretory function, we subjected 7-month-old mice (n = 7/genotype) to hyperglycemic clamp analysis. Maintaining a steady hyperglycemic state (at ∼320 mg/dl) in mutants and controls (Fig. 2C, panel i) required a comparable glucose infusion rate in both groups (Fig. 2C, panel ii). However, Cc2−/− mice displayed higher plasma insulin levels, suggesting preserved insulin secretory function (Fig. 2C, panel iii).

The increase in insulin levels in response to glucose does not appear to be compensatory to insulin resistance. Consistent with previous reports on 5-month-old mice (Patel et al., 2012), a 2-h hyperinsulinemic-euglycemic clamp analysis in overnight-fasted awake mice demonstrated that insulin sensitivity was maintained even at 7–8 months of age (Fig. 3).

Basal glucose at the beginning of the clamp (Fig. 3A) and basal hepatic glucose production (Fig. 3B), a measure of rate of appearance at pre-clamp condition, were similar in both groups of mice (Fig. 3B, vertical and patterned hatchings). During the clamp, at similar glucose levels (Fig. 3A, white and black bars), the glucose infusion rate required to maintain euglycemia was comparable in both groups of mice (Fig. 3C), indicating preserved systemic insulin sensitivity in the mutants, as was the case for 5-month-old male mice (Patel et al., 2012). Ceacam2 null mutation did not affect the ability of insulin to suppress hepatic glucose production (Fig. 3B, white and black bars) and promote whole body glucose disposal (turnover) (Fig. 3D, black versus white bars), including glucose uptake in gastrocnemius muscle (Fig. 3E). Insulin suppresses hepatic glucose production by inhibiting gluconeogenesis and stimulating net hepatic glucose uptake followed by glycogen synthesis (DeFronzo 1988). Consistently, whole body
glycolysis (Fig. 3F) and glycogen synthesis (Fig. 3G) were comparable in both groups of mice.

**CEACAM2 Regulates Release of Glucagon-like Peptide-1**—Because the distal part of the small intestine harbors cells that produce GLP-1 (Shalev 1997, Campbell et al., 2013), we then reassessed CEACAM2 expression in this tissue and investigated whether its regulation of insulin secretion implicates GLP-1 production and/or release. Immunofluorescence analysis detected CEACAM2 in the villi lining the intestinal segment beginning with distal jejunum (Fig. 4, A–C, green). As reported previously (Robitaille et al., 1999), CEACAM2 was also detected in crypt cells (Fig. 4, A–C).

Relative to their wild-type counterparts, 5-month-old Cc2−/− mice released a significantly higher level of GLP-1 (Fig. 5A) as well as insulin during an oral glucose tolerance test (Fig. 5B, panel i). Although their basal blood glucose level was normal, they manifested more tolerance to exogenous oral glucose (Fig. 5B, panel ii), consistent with their insulin sensitivity.

Pretreating with exendin (9–39), a GLP-1 receptor antagonist, suppressed the effect of Ceacam2 deletion on insulin (Fig. 5B, panel iii) as well as glucose excursion (Fig. 5C, panel iv) in response to oral glucose. Together, this suggests that Ceacam2 deletion regulates insulin secretion, at least partly, via a GLP-1-mediated mechanism.

To further examine the effect of CEACAM2 on GLP-1 production and/or secretion, we then reduced its expression in murine GLUTag enteroendocrine cells by means of siRNA transfection. As Fig. 6 shows, reduction of Ceacam2 mRNA (Fig. 6A) and protein (Fig. 6B) levels by 50% significantly induced GLP-1 release into the media of siRNA relative
to scrambled-transfected cells (Scr), both basally and in response to 11 mM glucose (Fig. 6C). In contrast, reducing CEACAM2 content did not affect mRNA levels of cellular proglucagon (Fig. 6D). Together, the data point to modulation of GLP-1 secretion from intestinal cells independent of other potential confounding metabolic factors.

**CEACAM2 Regulates GLP-1 Secretion through L-type Voltage-gated Ca\(^{2+}\) Channels**—Because L-type voltage-dependent Ca\(^{2+}\) channels are implicated in GLP-1 release from GLUTag cells (Sidhu et al., 2000, Reimann et al., 2005), we investigated whether CEACAM2 regulates GLP-1 release through voltage-dependent Ca\(^{2+}\) channel (VDCC)-mediated changes in intracellular calcium. To this end, we transfected GLUTag cells with scrambled (Scr) and Ceacam2-specific siRNA oligos (siCc2) before loading them with Fura-2, a calcium indicator, and monitored 340/380 nm fluorescence in response to membrane depolarization evoked by application of physiological saline containing elevated KCl concentrations (10–50 mM). As shown in Fig. 7, applications of elevated KCl induced concentration-dependent cytosolic Ca\(^{2+}\) rises. Whereas KCl at 10 and 20 mM yielded comparable calcium influx in both control and knocked down cells, 50 mM KCl induced a more pronounced calcium rise in siCc2 than Scr cells, as shown in the ratio images and corresponding line traces (Fig. 7, A and C). In addition, repeated depolarization with 50 mM KCl revealed only a slight reduction in a second cytosolic Ca\(^{2+}\) rise suggesting that the VDCC-underlying Ca\(^{2+}\) entry was not robustly inactivated by our stimulus paradigm, as indicated by the ratio images and respective line traces (Fig. 7, B and D). Moreover, the amount of inactivation of entry was similar between control and knocked down groups.
To further identify the calcium channel that mediated depolarization-evoked intracellular Ca\textsuperscript{2+} rises, the L-type VDCC blockers, nicardipine and nifedipine at 10 µM each, were used to selectively inhibit Ca\textsuperscript{2+} entry in response to 50 mM KCl application. As Fig. 7, E and F, reveals, elevated KCl failed to induce a rise in cytosolic Ca\textsuperscript{2+} levels in the presence of nicardipine and nifedipine, respectively. This suggests that enhanced Ca\textsuperscript{2+} entry in siCc2 cells was mediated by L-type VDCC and that CEACAM2 may regulate GLP-1 secretion by modulating L-type channel expression or activity. Cumulative averaged data for these experiments are shown in Fig. 7G.

Glucose Regulates Ceacam2 Expression—We have previously reported that refeeding following an overnight fast markedly represses Ceacam2 mRNA levels (Heinrich et al., 2010). Thus, we investigated whether glucose and/or insulin modulate(s) Ceacam2 expression. To this end, we treated GLUTag cells with 3, 5.5, or 20 mM glucose for 15 or 60 min. As Fig. 8A, panel i, reveals, glucose (5.5 mM) decreased Ceacam2 mRNA levels by 50% compared with 3 mM within 15 min of treatment. Increasing glucose concentration and prolonging the treatment period did not further change Ceacam2 mRNA expression. Consistently, Western blot analysis reveals no effect of glucose (3–20 mM) on CEACAM2 protein level after 15 min of treatment. However, treatment with 20 mM, but not 3 mM, glucose for ≥60 min reduced CEACAM2 protein level by ∼50% (Fig. 8A, panel ii). In contrast to glucose, insulin (100 nM) treatment for 15 or 60 min failed to modulate Ceacam2 mRNA levels in these cells (Fig. 8B).
Discussion

CEACAM1 and CEACAM2 are highly homologous proteins with differential tissue and cellular distribution (Han et al., 2001, Zebhauser et al., 2005) that predict distinct functions. CEACAM1 enhances insulin action by promoting insulin clearance in liver without affecting insulin secretion despite its significant expression in pancreatic \( \beta \)-cells (DeAngelis et al., 2008). Emerging evidence shows that CEACAM2 regulates insulin action by reducing food intake and modulating energy expenditure (Patel et al., 2012). Using global \( Cc2^{-/-} \) null mice, the current studies unravel a novel role for CEACAM2 in inhibiting insulin secretion, at least partly by a GLP-1-dependent mechanism.

Despite predominant CEACAM2 expression in non-\( \beta \) pancreatic islets, its deletion did not affect either the islet areas or basal plasma glucagon and somatostatin levels. Together with preserved \( \beta \)-cell secretory function and normal insulin release in response to both glucose and KCl in isolated pooled islets, this points to an extra-pancreatic regulatory mechanism on insulin secretion brought about by \( Ceacam2 \) deletion.

Enhanced insulin excursion in response to oral glucose was accompanied by a higher induction of GLP-1 release in \( Cc2^{-/-} \) mice. Suppression of the differential effect of oral glucose on insulin secretion and glucose tolerance by the GLP-1 receptor antagonist provides further evidence that CEACAM2 can regulate insulin secretion through a GLP-1-dependent pathway. Because GLP-1 potentiates glucose-dependent insulin secretion from \( \beta \)-cells (D’Alessio et al., 1994), it is likely that \( Ceacam2 \) deletion caused an increase in glucose-stimulated insulin secretion, at least partly, by inducing GLP-1 release. This is supported by elevated GLP-1 content in the media of GLUTag enteroendocrine cells following siRNA-mediated knockdown of Ceacam2. The mechanistic underpinning of
the regulation of GLP-1 secretion by CEACAM2 awaits further investigation, but the positive effect of Ceacam2 deletion on Ca\(^{2+}\) entry via L-type VDCC suggests that CEACAM2 regulates GLP-1 secretion in part by reducing cellular Ca\(^{2+}\) entry, consistent with the role of this process in mediating GLP-1 release from GLUTag cells (Sidhu et al., 2000, Reimann et al., 2005).

Moreover, induction of GLP-1 release from GLUTag cells by Ceacam2 down-regulation suggests that elevated plasma GLP-1 levels in Cc2\(^{-/-}\) mice are independent of other potential confounding metabolic changes stemming from Ceacam2 deletion.

Of note, higher plasma insulin levels in Cc2\(^{-/-}\) than in wild-type mice during hyperglycemic clamp analysis point to the possibility that CEACAM2 can also affect insulin secretion independently of GLP-1. In light of CEACAM2 expression in the ventromedial hypothalamus (Heinrich et al., 2010), the glucose-sensing center in the brain (Levin et al., 2004), it is possible that hypothalamic CEACAM2 regulates insulin secretion centrally (Thorens 2010, Osundiji et al., 2012) and independently of GLP-1 release (Chan et al., 2013). Disturbance in this neuronally mediated mechanism may contribute to the higher excursion of insulin during hyperglycemic clamp analysis in Cc2\(^{-/-}\) mice.

We have shown that fasting induces and refeeding represses Ceacam2 mRNA levels (Heinrich et al., 2010). Based on these studies, it is reasonable to postulate that reduction of Ceacam2 expression during refeeding could result from the rise in glucose. This, in turn, could elevate GLP-1 and subsequently insulin secretion. To avoid hyperinsulinemia, secreted insulin would induce CEACAM1 phosphorylation to promote receptor-mediated insulin uptake (Formisano et al., 1995) and hepatic insulin clearance (DeAngelis et al.,
2008, Poy et al., 2002) to maintain normal insulin metabolism and action on glucose homeostasis. Given that GLP-1 prompts transition into the fasting state (Barrera et al., 2011), this may also constitute a negative feedback mechanism to ultimately recover CEACAM2 levels and limit insulin secretion. Of note, CEACAM2 is expressed in the kidney but not liver (Han et al., 2001, Zebhauser et al., 2005). Whether it plays a role in renal insulin clearance is not known but, given that insulin clearance occurs mostly in the liver where CEACAM1 expression dominates, insulin clearance is not expected to be directly affected by Ceacam2 deletion, and elevation in insulin clearance in Cc2−/− mice would be mediated by CEACAM1 phosphorylation in response to insulin in liver (Xu et al., 2009, DeAngelis et al., 2008, Poy et al., 2002) as well as kidney (Al-Share et al., 2015).

In conclusion, the data provide evidence for a novel role for CEACAM2 in the regulation of insulin secretion, at least partly, via GLP-1 release. Further studies are required to elucidate the underlying mechanisms, but the current data highlight a relevant role for CEACAM2 in GLP-1 release.
Author Contributions—S. S. G. researched data, designed and coordinated experiments, and wrote the manuscript. G. H. researched and analyzed data and reviewed the manuscript. S. G. L., V. P., S. B., P. R. P., A. M. D., T. D., S. K. R., Z. N. S., Y. L., D. Y. J., and T. K. researched data. S. E. oversaw the immunofluorescence analysis of intestinal cells. R. N. K. oversaw, discussed the localization of CEACAM2 to islets, and revised the manuscript. J. K. K. oversaw and discussed clamp analysis data. D. R. G. oversaw and discussed data of live cell fluorescence and intracellular Ca$^{2+}$ measurements and revised the manuscript. S. M. N. was responsible for study design, conceptualization, data analysis, results interpretation, and reviewing the manuscript. S. M. N. has full access to all the data of the study and takes responsibility for the integrity and accuracy of data analysis and the decision to submit and publish the manuscript.

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Figure Legends

FIGURE 1. Characterization of CEACAM2 expression in the endocrine pancreas. A, Ceacam2 mRNA levels were determined in triplicate in FACS-purified β-cells and non β-cells by qRT-PCR (n = 5 mice), normalized to β-actin (panel i), and measured relative to Ceacam1 mRNA (panel ii). Values were expressed as means ± S.E. *, p < 0.05 versus β-cells. B, liver tissues were removed from 5-month-old mice (n >3/genotype) and lysates were analyzed by 7% SDS-PAGE with sequential immunoblotting (Ib) with polyclonal anti-body against CEACAM1 followed by re-immunoblotting (ReIb) with α-tubulin antibody for protein normalization. C, RNA was extracted from pancreatic islets of these mice and reverse-transcribed using specific Ceacam1 and Ceacam2 primers, followed by PCR amplification and analysis by 1% agarose gel. Gels in B and C represent at least two independent experiments.

FIGURE 2. Effect of CEACAM2 on islet area, insulin secretion from isolated islets, and β-cell secretory function. A, pancreatic sections from 6-month-old mice (n = 4/genotype) were fixed and immunostained with antibodies against insulin, glucagon, somatostatin, polypeptide P (PP), and 3Ab (a mixture made up of the last three antibodies). Six sections/mouse were analyzed. Magnification X 40. B, islets were isolated from 4-month-old mice (n >3/genotype) by collagenase digestion followed by centrifugation over Histopaque gradient and culturing overnight in RPMI 1640 medium containing 5.5 nmol/ liter glucose. Insulin secretion was assayed by incubating 10 islets in Krebs buffer containing different concentrations of glucose or 20 mmol/liter KCl for 1 h. The amount of insulin secreted was normalized to DNA content, and values were expressed as means ± S.E. C, awake mice (n = 7, 7-month-old Cc2+/+ (○) and Cc2−/− (●))
were continuously infused with glucose to maintain hyperglycemia (panel i), before glucose infusion rate (mg/kg/min) (panel ii), and plasma insulin (milliunits/ml) levels (panel iii) were determined. Values were expressed as means ± S.E. *, p <0.05 versus Cc2+/−.

FIGURE 3. Insulin sensitivity in awake mice. Seven-to-eight month-old overnight fasted awake mice (n = 11/genotype) were subjected to hyperinsulinemic-euglycemic clamp analysis. A, whole blood glucose. B, hepatic glucose production at basal (vertical hatchings, Cc2+/−; patterned hatchings, Cc2−/−) and clamp (white, Cc2+/−; black, Cc2−/−) conditions. *, p <0.05 versus basal. C, steady-state glucose infusion rate during clamp. D, whole body (WB) glucose turnover (Ra). E, glucose uptake in gastrocnemius muscle. F, whole body glycolysis. G, whole body glycogen synthesis (white, Cc2+/−; black, Cc2−/−). Values were all expressed as means ± S.E. in mg/kg.min−1 except for blood glucose levels (mg/dl).

FIGURE 4. Immunofluorescence analysis of intestinal CEACAM2 expression. Small intestinal sections from 5-month-old mice were fixed in Bouin’s solution and embedded in paraffin blocks prior to exposure to antigen retrieval and overnight incubation with α-CEACAM2 polyclonal antibody followed by Cy5 for visualization of CEACAM2 (green) in villi of distal jejunum/ proximal ileum (A–C) and in crypts (A –C). DAPI was used to visualize nuclei (D–F). Analysis was performed using a Keyence BZ9000. Scale bar, 100 µm.

FIGURE 5. Effect of CEACAM2 on GLP-1 and insulin release during an oral glucose tolerance test. A, plasma GLP-1 levels were measured from overnight fasted mice (n ≥6/genotype) following an oral administration of glucose. Values were expressed
as means ± S.E. *, p <0.05 versus Cc2+/+. B, plasma insulin concentrations (panel i) and blood glucose levels (panel ii) at the 0–120-min time period were determined in overnight fasted male 5-month-old Cc2+/+ (○) and Cc2−/− mice (●) mice after oral administration of glucose. Plasma insulin (panel iii) and blood glucose (panel iv) were also assessed in the presence of exendin (9–39), a GLP-1 receptor antagonist. n >5 mice/genotype. Values were expressed as means ± S.E. *, p <0.05 versus Cc2+/+.

FIGURE 6. Effect of CEACAM2 on GLP-1 secretion in GLUTag cells. GLUTag cells were transfected with scrambled (Scr-Cc2) or Ceacam2-specific siRNA (siRNA-Cc2). A, Ceacam2 mRNA; B, protein levels were then evaluated by qRT-PCR and Western blot analysis, respectively. Ib, immunoblot. C, GLP-1 secretion was assessed as percentage GLP-1 level in media relative to the sum of GLP-1 in media plus cell lysates. D, proglucagon mRNA levels were assessed by qRT-PCR. Values were expressed as means ± S.E. *, p <0.05 versus scrambled/treatment group; †, p <0.05 versus basal/transfection group.

FIGURE 7. CEACAM2 regulates GLP-1 secretion through L-type voltage-dependent Ca²⁺ channels. A and B, GLUTag cells were loaded with Fura-2 AM, and images were obtained. The ratio images illustrate [Ca²⁺]ᵢ rises in individual cells in a field of view following membrane depolarization evoked by applying elevated concentrations of KCl (10, 20, and 50 mM) (A) and two sequential stimulations with 50 mM KCl (B) in both scrambled (Scr) and si-RNA Ceacam2 transfected (siCc2) cells. C–F, representative line traces from cells in A and B (indicated by white circles) depict [Ca²⁺]ᵢ responses in GLUTag cells evoked by KCl (10, 20, and 50 mM) or sequential applications of 50 mM KCl. To assess VDCC contribution to the depolarization-evoked rises in cytosolic Ca²⁺,
50 mM KCl was applied prior to and following treatment with 10 µM of the L-type calcium channel blockers, nicardipine (NIC) and nifedipine (NIF), as indicated. Scale bars indicate fluorescence ratio units (r.u.) and time in seconds. G, bar graphs of the averaged changes in [Ca$^{2+}$]$_i$ in GLUTag cells stimulated by physiological saline containing the indicated concentrations of KCl (10, 20, and 50 mM) (panel i) or following treatment with nicardipine (panel ii) and nifedipine (panel iii). *, $p < 0.05$ versus 10 – 20 mM KCl/transfection group (i) and KCl/transfection group (ii), †, $p < 0.05$ versus scrambled group.

FIGURE 8. Effect of glucose and insulin on Ceacam2 expression. A, Ceacam2 mRNA level was measured in GLUTag cells in response to glucose (3–20 mM) for 15 and 60 min (panel i). Values were expressed as mean ± S.E. *, $p < 0.05$ versus 3 mM glucose. Panel ii, CEACAM2 protein levels were evaluated in GLUTag cells in response to glucose (3 and 20 mM) for 15, 60, and 120 min. Ib, immunoblot. B, as in A, except for stimulation with insulin (100 nM). Values were expressed as means ± S.E.
References


Table 1

Plasma biochemistry

Five-month-old male mice (n ≥13 for all parameters, except for n≥4 for GLP-1) were fasted overnight before retro-orbital blood was drawn, and plasma levels of hormones were assayed. To determine basal glucagon level, mice were fasted for 4 h before their retro-orbital blood was drawn. Data are means ± S.E.

<table>
<thead>
<tr>
<th></th>
<th>Cc2+/+</th>
<th>Cc2−/−</th>
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<tbody>
<tr>
<td>Body Weight (g)</td>
<td>25.7 ± 0.34</td>
<td>26.0 ± 0.65</td>
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<tr>
<td>Insulin (pM)</td>
<td>57.3 ± 4.19</td>
<td>63.0 ± 5.77</td>
</tr>
<tr>
<td>C-peptide (pM)</td>
<td>349. ± 38.3</td>
<td>670. ± 91.5  a</td>
</tr>
<tr>
<td>C/I ratio (steady state)</td>
<td>5.98 ± 0.44</td>
<td>10.3 ± 0.96  a</td>
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<tr>
<td>Glucagon (pg/ml)</td>
<td>57.9 ± 1.20</td>
<td>60.0 ± 1.28</td>
</tr>
<tr>
<td>Somatostatin (pg/ml)</td>
<td>819. ± 9.86</td>
<td>823. ± 16.1</td>
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<tr>
<td>GLP-1 (pM)</td>
<td>1.46 ± 0.15</td>
<td>4.81 ± 1.23  a</td>
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</table>

a p< 0.05 versus Cc2+/+.
Figure 1

A. RT-PCR in sorted mouse islets

\[ \text{i. Cc2 mRNA (\beta\text{-Actin})} \]

\[ \beta\text{-Cells} \quad \text{Non} \quad \beta\text{-Cells} \]

\[ \text{Relative Cc1/Cc2} \]

\[ \beta\text{-Cells} \quad \text{Non} \quad \beta\text{-Cells} \]

B. Western Analysis of Liver Proteins

\[ \text{Cc2}^{+/+} \quad \text{Cc2}^{-/-} \]

\[ \text{Mouse} \]

\[ \text{lb: } \alpha\text{-CC1} \]

\[ \text{Relb: } \alpha\text{-Tubulin} \]

C. RT-PCR Analysis of Pancreatic Islets

RT Primers: Ceacam1 Ceacam2

PCR Primers: Cc1 Cc2 Cc1 Cc2

Cc2 mouse

Cc1

Cc2
A. Immunohistochemical analysis of islets

B. Insulin secretion in pooled islets

C. Hyperglycemic clamp analysis
Hyperinsulinemic-Euglycemic Clamp

A. Blood Glucose (mg/dl)

B. Hepatic Glucose Production

C. Glucose Infusion Rate

D. WB Glucose Turnover (Rcl)

E. Gastrocnemius Glucose Uptake

F. WB Glycolysis

G. Glycogen Synthesis
Immunofluorescence analysis of CEACAM2 in the small intestine
Figure 5

A. Plasma GLP-1 levels post-oral glucose

B. Insulin release during oral glucose tolerance
Figure 6

GLP-1 secretion in GLUTag cells

A. Cc2 mRNA/18S

B. CEACAM2 protein levels

C. GLP-1 secretion (% media/medium+cell)

D. Proglucagon mRNA/18S
Figure 7

A. siCoc2
Scr

B. siCoc2
Scr

C.

D.

E. KCl

F. KCl

G. i. Δ[Ca²⁺] (x10⁻² nM)

• Scr
• siCoc2

ii. Δ[Ca²⁺] (x10⁻² nM)

KCL
NIC
KCL
NIC

iii. Δ[Ca²⁺] (x10⁻² nM)

KCL
NIF
KCL
NIF
A. Glucose Regulation of Ceacam2 Expression

i. Cc2 mRNA (/18S)

<table>
<thead>
<tr>
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<tr>
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</tr>
<tr>
<td>5.5 mM</td>
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</tr>
<tr>
<td>20 mM</td>
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* indicates significant difference

ii.

<table>
<thead>
<tr>
<th></th>
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<th>120'</th>
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<td>3</td>
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<tr>
<td></td>
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<td>3</td>
<td>20</td>
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</tr>
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Western Blotting:

- Igα-mCC2: 52 kDa
- Igα-GAPDH: 37 kDa

B. Insulin Regulation of Ceacam2 Expression

<table>
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<th></th>
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</thead>
<tbody>
<tr>
<td>Cc2 mRNA (/18S)</td>
<td>1.0</td>
<td>0.8</td>
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</tr>
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</table>
Chapter 3

The Role of CEACAM2 in Renal Insulin Clearance

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Nonstandard abbreviations used. CEACAM1, Carcinoembryonic antigen-related cell adhesion molecule 1; Ceacam1, murine gene encoding CEACAM1 protein; CEACAM2, Carcinoembryonic antigen-related cell adhesion molecule 2; Ceacam2, murine gene encoding CEACAM2 protein; Cc1+/−, global Ceacam1 null mouse; Cc2+/−, global Ceacam2 null mouse; BL6, C57BL/6J genetic background; Cc1+/+ and Cc2+/+, wild-type mice for both Ceacam1 and Ceacam2; KPTs, kidney proximal tubules.
Abstract

Consistent with its elevated hepatic expression, CEACAM1 promotes insulin clearance, which occurs mostly in liver and to a lower extent in kidney. This protein is expressed in proximal tubule cells of the rat and mouse. The homolog of CEACAM1, CEACAM2, is expressed in kidney but not liver, indicating a possible role of CEACAM2 in renal insulin disposal. Immunohistochemical analysis revealed expression of both CEACAM proteins in proximal tubular cells, a main site of renal insulin extraction. Since the intracellular phosphorylation sites are needed for receptor-mediated insulin uptake, we have reported the presence of long isoforms of both CEACAMs (CEACAM2-2L, CEACAM1-4L) in proximal tubule cells with CEACAM2-2L being more predominant. Supporting our hypothesis, insulin signaling activation mediated by phosphorylation of insulin receptor beta showed a decrease in kidney lysates of mutants during fed state and in proximal tubules from mutants upon insulin stimulation, in addition $[^{125}\text{I}]$ insulin internalization was lower in proximal tubular cells isolated from $Cc2^{−/−}$ mice compared to wildtype. These findings demonstrate that CEACAM2 mediates insulin clearance in kidney.
Introduction

The two transmembrane glycoproteins, CEACAM1 and CEACAM2, belong to the same carcinoembryonic antigen (CEA) family of proteins sharing high homology in their amino acid sequence, particularly in their intracellular domain, which accounts more than 95% homology (Han et al., 2001, Zebhauser et al., 2005, Robitaille et al., 1999). CEACAM1 is highly conserved among species (Prall et al., 1996, Estrera et al., 1999) with its gene composed of nine exons, the seventh undergoes alternative splicing to yield two variants, the long (CEACAM1-4L) and short isoform (CEACAM1-4S), each containing 4 IgG loops in the extracellular domain (Najjar et al., 1993). As opposed to the short isoform, the intracellular domain of CEACAM1-4L contains tyrosine and serine phosphorylation sites (Najjar et al., 1995). CEACAM1 promotes insulin clearance and maintains insulin sensitivity by enhancing receptor-mediated insulin internalization and degradation, after being phosphorylated by the insulin receptor tyrosine kinase mostly in liver and to a lower extent in kidney, consistent with its predominant hepatic expression (Poy et al., 2002, DeAngelis et al., 2008, Xu et al., 2009). Mice with global Ceacam1 null deletion (Cc1−/−) or its liver-specific inactivation (L-SACC1) exhibit hyperinsulinemia resulting from impaired insulin clearance that eventually causes increased hepatic lipogenesis and redistribution to adipose tissue, leading to visceral obesity in these mice (DeAngelis et al., 2008, Dai et al., 2004).

CEACAM2 is a close related protein to CEACAM1, with its gene encoded on chromosome 7 in close proximity to Ceacam1. It shares high homology with Ceacam1 of ~79.6% overall and 95.9% in the sequence encoding the intracellular domain with conserved tyrosine and serine phosphorylation sites (Han et al., 2001). Exons 3, 4, and 7
undergo alternative splicing to give CEACAM2 proteins with 2 IgG loops instead of 4 in the extracellular domain in either the short or long isoforms (CEACAM2-2S and -2L).

CEACAM2 has a limited tissue distribution compared to CEACAM1, the latter being ubiquitously expressed (except for skeletal muscle and white adipose tissue). CEACAM2 is expressed in kidney, testis, spleen, platelets, crypt epithelial cells of the small intestine, and several brain nuclei, including the ventromedial hypothalamus (Han et al., 2001, Zehhauser et al., 2005, Nedellec et al., 1994, Heinrich et al., 2010). This unique tissue distribution reflects its specific functions in central regulation of energy balance, brown adipogenesis, insulin secretion, platelet activation, thrombus stability and spermatid maturation (Heinrich et al., 2010, Patel et al., 2012, Alshahrani et al., 2014, Salaheldeen et al., 2012, Ghanem 2016).

Knowing that CEACAM1 plays an important role in hepatic insulin clearance consistent with its high expression in liver, this does not exclude a potential role of CEACAM2 in renal insulin clearance, as it is expressed in the kidney. Thus in our current studies, we investigate whether CEACAM2 is involved in renal insulin clearance, independently of CEACAM1.

Materials and Methods

Animals. The generation of global Ceacam1 and Ceacam2 null mice has been previously described (DeAngelis et al., 2008, Heinrich et al., 2010, Patel et al., 2012, Leung et al., 2006). Both lines were propagated onto the C57BL/6 genetic background. Male mice were kept in a 12-hour light–dark cycle having free access to standard chow.
Experimental protocols were approved by the Institutional Animal Care and Use Committee.

**Semi-quantitative Real Time-PCR (qRT-PCR).** Two month old $Cc1^{+/+}$, $Cc2^{+/+}$, $Cc1^{--}$, and $Cc2^{--}$ mice ($N=4$ per each group) were fasted and refed for 7 hours. Under general anesthesia by sodium-pentobarbital (50mg/ml), kidneys were collected and processed for mRNA expression of *Ceacam1* and *Ceacam2*. Expression of short and long isoforms of *Ceacam1* and *Ceacam2* (*Ceacam1-4L, Ceacam1-4S, Ceacam2-2L, Ceacam2-2S*) were also studied from isolated proximal tubule cells from 6-month old $Cc2^{+/+}$ and $Cc2^{--}$ mice. Studying gene expression was done by real time qPCR (ABI StepOnePlus Real-Time PCR System, Applied Biosystems, Foster City, CA). Total RNA was extracted from primary proximal tubule cells and frozen kidneys using PerfectPure RNA Tissue Kit-50 (5 PRIME-2900317-Inc., MD, USA). cDNA templates for qRT-PCR were synthesized using 1µg of total RNA, 5X iScript Reaction Mix including dNTPs, Random Primers, RNase inhibitor and iScript reverse transcriptase RNase H+ (Biorad iScript cDNA Synthesis Kit 170-8891). qRT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA).

**Immunohistochemistry (IHC).** Age-matched $Cc2^{+/+}$ and $Cc2^{--}$ mice were anesthetized with sodium-pentobarbital (50mg/ml). Kidneys were collected and fixed in 10% neutral buffered formalin (EMD 65346-88) for 24 hours and then stored in 70% Ethanol. Sections including the cortex and medulla were immunostained with custom-made anti-CEACAM1 (α-mCC1) (DeAngelis et al., 2008) or anti-CEACAM2 (α-mCC2) antibody (anti-peptide polyclonal antibody raised in rabbit against the KLH-conjugated HPLC purified peptide CNAEIVRFVTGNTIKGPVH in CEACAM2; Bethyl Laboratories,
Primary kidney proximal tubule cells. Proximal tubule cells were isolated from kidneys of 6-month old anesthetized mice (N=7/per each strain). After kidney decapsulation, they were cut horizontally, and the medulla discarded while cortex was finely divided by a razor blade in 1ml of solution 1 (DMEM-F12, 1mM Heptanoate Acid, 4mM Glycine, pH 7.4). Cortical fragments were digested three times (shaking at 37°C in 100% oxygen for 12 min) in 10ml of Collagenase Solution [(1mg/ml) Collagenase type II (Worthington), 1mg/ml insulin-free BSA, 0.1mg/ml DNAse I (Sigma)], and allowed to settle down by gravity into a 10ml sterile pipette. The final combined supernatant was centrifuged at 1000rpm at 4°C for 5 min to collect cell pellet that was reconstituted in Percoll Solution (Sigma), and ultracentrifuged in Nalgene tubes (Thermo Scientific) at 17000rpm at 4°C for 30 min. Proximal tubular cells aggregating in between the third and fourth layer at ~1.0-1.3g/ml Percoll density were carefully removed and washed twice in 50ml of Solution 1 (1000rpm, 4°C, 5 min) in 50ml falcon tube. They were combined and plated at equal density onto poly L-lysine coated 6-well-plates at 37°C in a humidified 5% CO2-95% air incubator for 1-2 days in DMEM/F-12 culture medium (Sigma), containing 1% penicillin-streptomycin, 60nM sodium selenite, 1.1mg/ml sodium bicarbonate, 5µg/ml human apo-transferrin, 2mM glutamine, 50nM dexamethasone, 5pM 3,5,3’-triiodothyronine, 25ng/ml Prostaglandin E1, 50nM hydrocortisone, 10ng/ml epidermal growth factor, 5µg/ml insulin, 3.1g/l D-glucose, 2% (vol/vol) FBS, and 20mM Hepes, pH 7.4 (all from Invitrogen and Sigma). Cells were kept in the culture medium for a total of 24-48 hr.
**Ligand binding and internalization.** Isolated proximal tubule cells were isolated from *Cc2*<sup>+</sup>/ and *Cc2*<sup>−/−</sup> mice and grown for 36 hours prior to binding of 30,000cpm [I<sup>125</sup>] insulin (Human I<sup>125</sup>insulin, Perkin-Elmer). Insulin internalization was assessed, as previously described (Poy et al., 2002).

**Biotin labeling of surface membrane proteins.** Proximal tubule cells isolated from *Cc2*<sup>+</sup>/ and *Cc2*<sup>−/−</sup> mice were grown for 24 hours prior replacing DMEM/F-12 media with its low glucose dose (2.5mM) for 30 minutes in order to maintain normal expression of *Ceacam2*. These cells were first incubated in the absence or presence of 100nM insulin at 37°C for 5 min, followed by incubation with biotin (1mg/ml) (Pierce) for 30 min on ice to study the internalization. To study activation, cells were first biotin labeled for 30 min on ice and then stimulated with 100nM insulin at 37°C for 5 min. Following these steps, cells were washed with PBS (with Ca<sup>2+</sup> and Mg<sup>2+</sup>-100mg/L each and 15mM glycine) to be lysed afterwards. Proteins were immunoprecipitated with streptavidin (Pierce) overnight at 4°C. Immunoprecipitated complexes were isolated by centrifugation, washed three times with 1X PBS, and eluted with 1X Laemmli sample buffer (Biorad).

**Western Blotting.** Proteins from biotinylated proximal tubule cells and kidney lysates were electrophoresed onto a 7% SDS-PAGE and immunoblotted with polyclonal antibodies against the alpha subunit of the insulin receptor (IRα) (N20, Santa Cruz), phospho-insulin receptor beta (pIRβ) (phospho-Y1361) (Abcam), IRβ (C18C4) (Abcam), anti-phospho-CEACAM1 (α-pCC1) and custom-made polyclonal antibodies against mouse CEACAM1 (α-mCC1 Ab3759) (DeAngelis et al., 2008), and CEACAM2 (α-mCC2). Blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG antibody and donkey anti-rabbit IgG antibody (GE Healthcare Life Sciences,
Amersham), followed by enhanced chemiluminescence (ECL, Amersham Pharmacia).

**Statistical analysis.** Quantitative data were presented as mean ± standard error of the mean (SEM). Values were analyzed by One-way ANOVA or student’s t test. *P<0.05* was considered significant.
Results

*Ceacam1* and *Ceacam2* gene expression were measured in kidneys isolated from wild-type, *Cc1*+/−, *Cc2*−/− mice by semi-quantitative Real Time PCR analysis (Figure 1A). *Ceacam1* was expressed in the fed state of *Cc1*+/+ and *Cc2*−/− mice with no significant difference. As a negative control, *Ceacam1* was not detectable in kidneys of *Ceacam1* knockout mice. On the contrary of *Ceacam1*, *Ceacam2* was expressed in *Cc2*+/+ and *Cc1*−/− mice at fasting state. No detectable expression of *Ceacam2* was found in *Cc2*−/− mice. Taken this data together, *Ceacam1* and *Ceacam2* are both expressed in the kidneys being predominant in the fed and fasting state respectively (Fig 1A).

Localization of CEACAM proteins was determined in kidney sections by immunohistochemistry, which showed that CEACAM1 is in cortex (Figure 1B i versus ii -left panel) and medulla (Figure 1B iii versus iv -left panel). CEACAM1 expression (brown staining) was restricted to epithelial cells of proximal tubule cells and bowman capsule of the renal cortex in wild-type mice (Figure 1B i) and *Cc2*−/− mice (Figure 1B ii) indicating that CEACAM1 stays intact in CEACAM2 mutant mice. It was not detected in vessels of renal corpuscle, distal tubules and peritubular vessels (Figure 1B i versus ii). In the renal medulla, immunostaining showed that CEACAM1 is localized in the thin segment of Henle loop (Figure 1B iii) with no expression in the ascending part of distal tubules or collecting duct of peritubular vessels (Figure 1B iii).

Immunostaining for CEACAM2 (Figure 1B-right panel) showed that wild-type mice also expressed CEACAM2 in cortex (Figure 1B i) and medulla (Figure 1B ii). In renal cortex, CEACAM2 was only visible (brown staining) in epithelial cells of proximal renal tubules
No specific staining was found in sections derived from CEACAM2 knockout mice, as negative control (Figure 1B ii). Unlike CEACAM1, CEACAM2 was not found in bowman capsule (Figure 1B i). Comparable to CEACAM1, no specific staining for CEACAM2 was visualized in vessels of renal corpuscle, distal tubules and peritubular vessels neither of wild-type mice (Figure 1B i) nor CEACAM2 knockout mice (Figure 1B ii). In addition, immunostaining of renal medulla showed that CEACAM2 was expressed in the thin segment of Henle loop (Figure 1B iii), similar to CEACAM1. No detectable CEACAM2 was in the ascending part of distal tubules and in collecting duct of peritubular vessels (Figure 1B iii). As a control there was no staining in mutant mice (Figure 1B iv).

To further study the prevalent isoforms of CEACAM proteins in the kidney, the expression of short and long isoforms in isolated proximal tubules was analyzed. As shown in Figure 2, both isoforms of each gene were found, with Ceacam2-2L and Ceacam2-2S more abundant than Ceacam1-4L and Ceacam1-2S. These results indicate that the gene expression encoding the active form of CEACAM2 (Ceacam2-2L) is more abundant than that of CEACAM1 (Ceacam1-4L).

Insulin signaling was studied in kidney lysates from Cc2+/+ and Cc2-/- mice under fasting and refed states. As shown in Fig 3A, CEACAM1 phosphorylation was intact in both wildtype and mutant mice upon refeeding indicating that CEACAM2 deletion has no effect on CEACAM1 action in insulin disposal. CEACAM2 phosphorylation showed an increase upon refeeding in wildtype mice only; on the other hand, it was abolished in the knockout mice. Insulin receptor beta signaling was much higher in wildtype mice during
the feeding state compared to the mutant mice indicating that both CEACAM proteins play roles in renal insulin clearance (Fig 3A). These data further support the idea that CEACAM2 is a substrate of the insulin receptor and both CEACAM proteins have independent roles in insulin internalization in kidney. The expression of CEACAM1 and CEACAM2 in proximal tubule cells and the importance of these cells in insulin uptake and degradation made us investigate the role of CEACAM2 in regulating insulin clearance by kidney proximal tubule cells. Compared to wildtype mice, $^{125}$I-insulin internalization in primary tubule cells isolated from Cc2$^{-/-}$ mice was markedly lower (Figure 3B i), suggesting that CEACAM2 deletion diminished renal insulin clearance in vivo. This gives strong evidence that kidney proximal tubules lacking CEACAM2 were not capable to internalize insulin at a normal extent, attributing to a physiological role of CEACAM2 in the regulation of insulin clearance in kidney.

Consistent with the fact that both CEACAM proteins are highly homologous especially in the intracellular domain, we aimed to determine whether CEACAM2 is a part of the insulin internalization machinery specifically in KPT cells. Thus, the rate of insulin internalization in KPTs was assessed by biotin labeling of surface membrane proteins, including insulin receptor alpha (IR$\alpha$), CEACAM1 and CEACAM2 followed by streptavidin-mediated immunoprecipitation and immunoblotting. As shown in Figure 3B ii, KPT cells from Cc2$^{+/+}$ mice, when stimulated with insulin, internalized more IR$\alpha$. On the contrary, cells from Cc2$^{-/-}$ mice showed higher surface-biotin-labeled IR$\alpha$, which corresponded to less cellular endocytosis. Based on the fact that CEACAM1 expression is intact in KPTs of Ceacam2 mutant mice, we also determined the rate of internalization of CEACAM1, which showed comparable internalization in Cc2$^{+/+}$ and Cc2$^{-/-}$ isolated
KPT indicating its role in renal insulin clearance independent of CEACAM2. Similarly, CEACAM2 was only internalized in insulin-stimulated wildtype KPT cells compared to its control knockout KPTs, supporting the novel notion of CEACAM2 role in renal insulin disposal. Insulin signaling indicated by phosphorylation of insulin receptor beta decreased in mutant KPTs (Fig 3Biii), this was mediated by the loss of CEACAM2. CEACAM1 phosphorylation (pCC1) was intact in insulin stimulated cells from Cc2+/+ and Cc2−/− mice suggesting that ablation of CEACAM2 did not affect receptor-mediated insulin clearance of CEACAM1. Similarly, CEACAM2 was only activated and phosphorylated in response to insulin in Cc2+/+ KPTs in greater extent than CEACAM1 explaining the higher expression of its long isoform (CEACAM2-2L).

The expression of Ceacam1 and Ceacam2 was monitored in KPTs isolated from Cc2+/+ and Cc2−/− mice, as shown in Supplementary Figure S1 A, indicating that the deletion of Ceacam2 did not affect the expression of Ceacam1. As a control and to further verify that KPTs undergo insulin uptake, kidney derived cells from wildtype mice were studied and exhibited normal insulin internalization within time (Supplementary Figure S1 B).

**Discussion**

The two main sites for insulin clearance are the liver and kidney, the first organ clearing more than 50% of insulin during its first passage in portal vein directly following its secretion from beta pancreatic cells (Sato et al., 1991, duckworth et al., 1988). The process of insulin clearance in the liver occurs mainly through the transmembrane glycoprotein, CEACAM1, which undergoes phosphorylation in response to insulin leading to receptor mediated insulin endocytosis and degradation in hepatocytes (Najjar
et al., 1995, Poy et al., 2002, DeAngelis et al., 2008, Accili et al., 1986, Formisano et al., 1995). On the other hand, the kidney is responsible in removing approximately 30-40% of circulating peripheral insulin thus giving it an essential role in insulin action and degradation (Rabkin et al., 1983, Rabkin et al., 1984, Duckworth et al., 1998). Insulin in the kidney undergoes filtration by the glomerulus and further uptake and degradation in the cells lining the proximal convoluted tubules (Rabkin et al., 1983, Hysing et al., 1989, Rubenstein et al., 1968).

Despite the great similarity of CEACAM1 and CEACAM2 proteins in their gene sequence and protein structure (Han et al., 2001, Zebhauser et al., 2005), previous studies have shown differences in their tissue distribution reflecting unique functions (Poy et al., 2002, Heinrich et al., 2010, Patel et al., 2012, Alshahrani et al., 2014, Salaheldeen et al., 2012, Ghanem et al., 2016). However, if these proteins are expressed in same cell type, there is emerging data showing that they can share similar functions such as the case of platelets where CEACAM1 and CEACAM2 play independent roles in platelet activation (Alshahrani et al., 2014). Thus, in the current studies we investigate the possible role of CEACAM2 specifically in renal insulin disposal. According to our immunohistochemical analysis on kidney sections in this report and consistent with previous findings demonstrating the expression of both molecules in the kidney (Nedellec et al., 1994, Heinrich et al., 2010, Gupta et al., 2012), we show that CEACAM proteins share identical localization in the epithelia of proximal tubules and in the thin segment of Henle loop of the nephron, which are primary sites for insulin clearance in the kidney (Nielsen 1992). In this study, we also report that CEACAM1 expression in the kidney was higher in the
fed state compared to CEACAM2 that was more prevalent in the fasting metabolic state, consistent with previous reports (Heinrich et al., 2010, Ghanem et al., 2016).

These results show a decrease in insulin receptor beta phosphorylation in kidney lysates from mutant mice compared to wildtype upon refeeding; reinforcing that CEACAM2 is an insulin receptor substrate. This is consistent with the fact that proximal tubule cells express the active isoforms, CEACAM2-2L and CEACAM1-4L that harbor essential phosphorylation sites in the intracellular domain necessary for insulin internalization (Choice et al., 1999). A reduction in insulin signaling, identified by less phosphorylation of insulin receptor beta in insulin stimulated cells from Cc2−/− mice, was reported. CEACAM1 phosphorylation was intact in insulin-stimulated cells from both groups with no effect of CEACAM2 ablation on its signaling. CEACAM2 activation and phosphorylation was only evident in insulin-stimulated cells from control mice to a larger extent than pCEACAM1 consistent with its greater expression of the active long isoform.

Knowing that proximal tubule cells are the primary site for renal insulin clearance after glomerular filtration and express CEACAM proteins, we show that both proteins have independent functions in insulin disposal and contribute equally to this process. Receptor-mediated [125I] insulin internalization in primary proximal tubule cells from wildtype and Cc2−/− mice support this idea. The results demonstrate an important role of CEACAM2 in insulin internalization since the null mutation of CEACAM2 shows a decrease in this process. Moreover, studying internalization of insulin receptor alpha showed higher biotinylated surface membrane protein expression in insulin treated cells from Cc2−/− mice compared to wildtype demonstrating lower insulin internalization in the mutant cells due to the CEACAM2 deletion. Moreover, CEACAM1 and CEACAM2 proteins
showed a similar trend of internalization in response to insulin stimulation as IRα. This indicates that both proteins have significant roles in renal insulin clearance and both work independently.

Given the importance of insulin clearance in maintaining normal insulin levels and enhancing insulin sensitivity, and the fact that a decrease in this process leads to hyperinsulinemia and insulin resistance, our current findings characterize the significance of CEACAM2 in renal insulin clearance. The importance of insulin clearance was highlighted by previous studies that CEACAM1 knockout mice are characterized with metabolic abnormalities, including high blood pressure, insulin resistance and hyperinsulinemia (DeAngelis et al., 2008, Huang et al., 2013). To conclude, we report an important role of CEACAM proteins in renal insulin clearance that can represent a therapeutic tool for cases of renal insulin clearance dysfunction-driven hyperinsulinemia.
Figure Legends

Figure 1. Expression and localization of CEACAM proteins in kidney. qRT-PCR analysis of Ceacam1 and Ceacam2 in kidney lysates (A) derived from Cc1\(^{+/+}\) and Cc2\(^{+/+}\) (white bars), Cc1\(^{-/-}\) (black bars) and Cc2\(^{-/-}\) mice (grey bars) during fasting and fed states (N=4/per each group). Samples were analyzed in duplicates and normalized to GAPDH. Values, are expressed as mean ± SEM. *p<0.05 versus each group/state. (B) Kidneys derived from Cc2\(^{+/+}\) and Cc2\(^{-/-}\) mice (males; N=3/per each group) were immunostained with α-mCC1 and α-mCC2 in cortex (B i-ii) and medulla (B iii-iv).

Figure 2. Expression of CEACAM isoforms. qRT-PCR analysis of long and short isoform of Ceacam genes in proximal tubule cells derived from Cc2\(^{+/+}\) (white bar), Cc2\(^{-/-}\) (black bar) (N=4/per each group; samples were analyzed in duplicates). Values, normalized to GAPDH, are expressed as mean ± SEM. *p<0.05 versus each group.

Figure 3. Ligand binding and internalization. (A) Kidney lysates from male Cc2\(^{+/+}\) and Cc2\(^{-/-}\) mice during fasting and refeeding states were analyzed and electrophoresed using antibodies against α-CEACAM1, α-CEACAM2, phosphoCEACAM1 (pCC1), phosphoCEACAM2 (pCC2), pIR-β, and IR-β. (B) Insulin internalization in proximal tubule cells (i) isolated from male Cc2\(^{+/+}\) (black line) and Cc2\(^{-/-}\) (dotted line) mice (males; N=7; per strain) Values are expressed as mean ± SEM. *p<0.05 versus Cc2\(^{+/+}\). Proximal tubules from male Cc2\(^{+/+}\) and Cc2\(^{-/-}\) mice were treated with or without insulin for 5 minutes before labeling cell-surface proteins with biotin (ii) and vice versa (iii). Proteins were immunoprecipitated by streptavidin and electrophoresed using antibodies.
against IR-α, α-CEACAM1, α-CEACAM2, phosphoCEACAM1 (pCC1), phosphoCEACAM2 (pCC2), and pIR-β.

**Figure S1.** (A) qRT-PCR analysis for *Ceacam2* and *Ceacam1* expression in proximal tubule cells from male *Cc2^{+/+}* (white bar) and *Cc2^{-/-}* (black bar) mice. Data was normalized to 18S. Values are expressed as mean ± SEM. *p<0.05 versus *Cc2^{+/+}. (B) Insulin internalization in proximal tubule cells isolated from wildtype mice (males; N=7) upon incubation with [^{125}_I] insulin. Values for each time-point after insulin internalization was tested in triplicates. Values are expressed as mean ± SEM.
References


### Table 1: Primers used in RT-PCR

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<tr>
<td>18S</td>
<td>TTCGAACGTCTGCCCCTATCAA</td>
<td>ATGTTAGGCACGCGACTA</td>
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</table>
Figure 1

A. Ceacam mRNA expression in kidney

![Bar graph showing mRNA expression of Ceacam1 and Ceacam2 in FED and FAST conditions for different genotypes: Cc1<sup>+</sup>, Cc1<sup>−/−</sup>, Cc2<sup>−/−</sup>, Cc2<sup>−/+</sup>, Cc2<sup>+/−</sup>, Cc2<sup>+/+</sup>.

B. CEACAM immunostaining in kidney

![Images showing CEACAM1 and CEACAM2 immunostaining for different genotypes: Cc2<sup>−/−</sup> and Cc2<sup>−/+</sup>.

---

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Figure 2

Long and Short Ceacam2 and Ceacam1 expression in proximal tubule cells
A. Insulin signaling in kidney lysates

B. Insulin Internalization in proximal tubule cells
Figure 4

A. Ceacam2 and Ceacam1 expression in proximal tubule cells

B. Insulin Internalization in proximal tubule cells from WT mice
Chapter 4

Age-dependent insulin resistance in male mice with null deletion of the carcinoembryonic antigen-related cell adhesion molecule 2 gene

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Abstract

Aims/hypothesis Cc2\(^{-/-}\) mice lacking the carcinoembryonic antigen related cell adhesion molecule 2 (CEACAM2) exhibit hyperphagia that leads to obesity and insulin resistance starting at 2 months of age in females. Male mutants maintain normal body weight and insulin sensitivity until the last age perviously examined (7-8 months), owing to increased sympathetic tone to white adipose tissue and energy expenditure. The current study investigates whether insulin resistance develops in mutant males at a later age and whether this is accompanied by changes in insulin homeostasis.

Methods Insulin response was assessed by insulin and glucose tolerance tests, and energy balance was analyzed by indirect calorimetry.

Results The current work demonstrates that male Cc2\(^{-/-}\) mice develop overt metabolic abnormalities at about 9 months of age. These include elevated global fat mass, hyperinsulinemia and insulin resistance (as determined by glucose and insulin intolerance, fed hyperglycemia and decreased insulin signaling pathways). Pair-feeding experiments show that insulin resistance results from hyperphagia and indirect calorimetry demonstrates that older mutant males develop compromised energy expenditure. Despite increased insulin secretion caused by Ceacam2 deletion, chronic hyperinsulinemia did not develop in mutant males until about 9 months of age, at which point insulin clearance begins to decline substantially, likely mediated by a marked decrease in hepatic Ceacam1 expression.

Conclusions/interpretation The data demonstrate that at about 9 months of age, Cc2\(^{-/-}\) male mice develop reduction in energy expenditure and energy imbalance, which
combined with a progressive decrease in CEACAM1-dependent hepatic insulin clearance, cause chronic hyperinsulinemia and sustained age-dependent insulin resistance. This represents a novel mechanistic underpinning of age-related impairment of hepatic insulin clearance.

**Keywords** CEACAM2; Insulin clearance; Insulin secretion; Hyperinsulinemia; Insulin resistance; Energy balance; Hyperphagia
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<td>Adbr</td>
<td>Adrenergic b receptor</td>
</tr>
<tr>
<td>Ceacam2</td>
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</tr>
<tr>
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<td>CEACAM</td>
<td>Carcinoembryonic antigen-related cell adhesion molecule</td>
</tr>
<tr>
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</tr>
<tr>
<td>CPT1</td>
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</tr>
<tr>
<td>DIO2</td>
<td>Type 2 deiodinase</td>
</tr>
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<td>Fasn</td>
<td>Fatty acid synthase in mice</td>
</tr>
<tr>
<td>FATP</td>
<td>Fatty acid transport protein</td>
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<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>IRβ</td>
<td>β-Subunit of the insulin receptor</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
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<td>Pyruvate dehydrogenate kinase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor α</td>
</tr>
<tr>
<td>SNA</td>
<td>Sympathetic nerve activity</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>--------------------------------------</td>
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<tr>
<td>UCP</td>
<td>Uncoupling proteins</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial nucleus of the hypothalamus</td>
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<td>$\dot{V}CO_2$</td>
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<td>$\dot{V}O_2$</td>
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**Introduction**

The carcinoembryonic antigen-related cell adhesion molecule 2 (CEACAM2) is a transmembrane glycoprotein that belongs to the carcinoembryonic antigen (CEA) family (Kuespert et al., 2006, Robitaille et al., 1999). Its gene is distinct from that encoding CEACAM1, another member of this family of proteins that is ubiquitously expressed (Najjar 2002) and that regulates insulin sensitivity by promoting insulin clearance (Poy et al., 2002, DeAngelis et al., 2008), which predominantly occurs in liver and to a lower extent in kidney (Najjar 2002).

CEACAM2 is expressed in kidney, spleen, testis and platelets in addition to the crypt cells and the villi lining of the intestinal segment beginning with distal jejunum (Heinrich et al., 2012, Salaheldeen et al., 2012, Alshahrani et al., 2014, Han et al., 2001, Nedellec et al., 1994, Zebhauser et al., 2005, Ghanem et al., 2016). It is also expressed in the hypothalamus, including the ventromedial hypothalamus (VMH), and other brain centers involved in feeding behavior, including hippocampus, striatum, olfactory bulb and the globus and ventral pallidus (Heinrich et al., 2012, Morton et al., 2006, Cota et al., 2006). Consistently, the metabolic phenotype of mice with null mutation of *Ceacam2* gene (*Cc2*−/−) shows that CEACAM2 is involved in the central regulation of food intake and energy expenditure, and in the sympathetic regulation of brown adipogenesis (Heinrich et al., 2012, Patel et al., 2012).

Whereas CEACAM2 is detected in pancreatic cells, in particular in non b-cells, its null deletion does not influence basal plasma levels of hormones (insulin, glucagon and somatostatin), nor does it affect islet areas or glucose-induced insulin secretion in pooled


Cc2−/− islets (Morton et al., 2006). Instead, CEACAM2 promotes glucose-stimulated insulin secretion, at least in part, via a mechanism dependent on glucagon-like peptide-1 (GLP-1), consistent with its expression in the distal intestinal villi (Morton et al., 2006). In addition to its role in metabolism, CEACAM2 regulates spermatid maturation (Salaheldeen et al., 2012, Salaheldeen et al., 2014) and platelet activation and adhesion (Alshahrani et al., 2014).

Both male and female Cc2−/− mice display hyperphagia. Given that plasma leptin level is normal at the onset of hyperphagia in both sexes, it is more reasonable to predict that hyperphagia in Cc2−/− mice is primarily caused by the absence of CEACAM2 from brain centers that control feeding behavior and rewards instead of changes in leptin sensitivity (Bingham et al., 2008). This notion is supported by induction of brain CEACAM2 production at fasting and its suppression at refeeding (Heinrich et al., 2010). Studies in GLUTag entero-endocrine cells revealed that glucose, but not insulin, rapidly suppresses Ceacam2 mRNA level at refeeding (Ghanem et al., 2016).

Hyperphagia causes obesity and insulin resistance in female Cc2−/− mice at 2 months of age, as demonstrated by pair-feeding experiments (Heinrich et al., 2010). In contrast, it fails to trigger insulin resistance in male mutants up to 7 months of age (Ghanem et al., 2016, Patel et al., 2012), likely due to the differential hypermetabolic state in relation to activated sympathetic tone regulation of white adipose tissue and increased spontaneous activity in male mutants (Patel et al., 2012). Cc2−/− males remain lean and insulin sensitive until about 7-8 months of age, as demonstrated by hyperinsulinemic-euglycemic clamp analysis (Ghanem et al., 2016). They also manifest elevated glucose-induced GLP-1–mediated insulin secretion with preserved b-cell insulin
secretory function at this age, as shown by hyperglycemic clamp analysis (Ghanem et al., 2016). Thus, the current study investigated whether chronic hyperphagia eventually causes insulin resistance in older male mice, and whether this is accompanied by changes in insulin homeostasis.

Methods

Animals

The generation of \( Cc2^{-/-} \) mice has been previously described (Heinrich et al., 2010). Male mice (5-12 months of age) were housed in a 12h-dark/light cycle and fed standard chow (Teklad 2016) \textit{ad libitum} and kept in specific pathogen-free conditions at the Division of Laboratory Animal Resources at the University of Toledo. The Institutional Animal Care and Utilization Committee (IACUC) approved all procedures and animal experiments. For fasting, mice were transferred from corn cob bedding to alpha-dri bedding (Shepherd).

Body composition

Whole body composition was assessed by Nuclear Magnetic Resonance technology (Bruker Minispec) (Al-Share et al., 2015).

Metabolic parameters

Retro-orbital venous blood was drawn at 1100h from overnight fasted mice under pentobarbital anesthesia (1.1 mg/kg body weight) to measure plasma insulin (80-
INSMSU-E01, ALPCO), C-peptide (80-CPTMS-E01, ALPCO), and total GLP-1 levels (GLP1T-36HK, Millipore). The steady-state C-peptide/Insulin molar ratio was calculated as a surrogate marker for insulin clearance (Poy et al., 2002, Al-Share et al., 2015).
Whole blood glucose measurements from tail snipping were made with a glucometer from overnight fasted and randomly fed mice (Accu-check, Roche). Plasma non-esterified fatty acids (NEFA-C; Wako) and triacylglycerol (Pointe Scientific) were also measured. Tissue triacylglycerol content was assayed, as described previously (Al-Share et al., 2015).

**Intra-peritoneal insulin tolerance test**
Animals were fasted for 5hrs starting at 700h before being subjected to an intraperitoneal (IP) injection of Human Insulin Novolin (Novo Nordisk NDC 0169-1833-11) at 0.75U/kg body weight. Blood glucose was measured from the tail vein at 0-180min post-insulin injection.

**Glucose tolerance test**
Five days after the insulin tolerance test, animals were fasted overnight from 1700h to 800h before receiving either an intraperitoneal injection (1.5g/kg BW) or an oral gavage (3g/kg BW) of glucose (50% dextrose solution). Blood glucose level was measured from the tail vein of awake mice at 0-120min post-glucose administration.

**Glycogen assay**
As previously described (Park et al., 2006), livers were removed from mice fasted and
refed for 7hrs, digested (100mg) in 6% Percholic Acid, and incubated in 1mg/ml amyloglucosidase for 2hrs at 42°C. Glucose assay reagent (Sigma) was added and samples were incubated at room temperature for 15min, analyzed at A$_{340}$ nm, and converted to µg/mg wet tissue weight.

**Western blot analysis**

Proteins were analyzed by 7%SDS-PAGE and immunoprobed with polyclonal antibodies (1:1000) against phospho-insulin receptor (pIRβ) (phospho-Y1361) and IRβ (C18C4) (Abcam), phospho-Ser473 Akt and Akt (Cell signaling), and custom-made polyclonal antibodies raised in rabbit against mouse CEACAM1 (α-mCC1 Ab3759) and phospho-CEACAM1 (α-pCC1) (Bethyl) as previously titrated and optimized (Al-Share et al., 2015). Blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit IgG antibody (GE Healthcare Life Sciences, Amersham), followed by enhanced chemiluminescence.

**Pair-feeding**

The average daily food intake was assessed in individually caged 9-month-old mice over a 5-day-period before mutants were subjected to a pair-feeding regimen: 3.2g of food/day (0.5g less than the *ad libitum* (AL)-fed Cc2°/−) for the first week, followed by 3.7g vs 5.5g of food/day to the AL-group in the second week.
**Insulin secretion**

Overnight fasted mice were anesthetized with pentobarbital (1.1mg/kg BW) before receiving an intraperitoneal glucose injection (3g/kg BW). Retro-orbital blood was drawn at 0-120min post-glucose injection to measure insulin levels.

**Immunofluorecence analysis**

Mice were anesthetized with pentobarbital (1.1mg/kg body weight), and pancreata were dissected, weighed, fixed in Z-fixative, sectioned and immunostained with anti-human insulin (Linco) and anti-mouse glucagon monoclonal antibodies (Sigma), followed by AMCA-conjugated donkey and Texas red–conjugated donkey antibody (Jackson ImmunoResearch), respectively (Morioka et al., 2007). α and β-Cell areas were calculated by morphometric analysis using Image J software (http://rsb.info.nih.gov/ij/) (Michael et al., 2000, Kulkarni et al., 2003), and the glucagon/insulin-stained area was divided by total pancreas area.

**Indirect calorimetry**

Indirect calorimetry was measured in awake mice (n=5/genotype), as described (Al-Share et al., 2015). Mice were individually caged (CLAMS system, Columbus Instrument) for 3 days after being acclimated for 2 days and had free access to food and water. Spontaneous locomotor activity was measured with an optical beam device quantifying horizontal and vertical movement (X-, Y- and Z axes). Oxygen consumption (VO₂) and heat production (energy expenditure-EE) were measured every 20min and data normalized per fat-free lean mass.
Quantitative real-time-PCR (qRT-PCR)

Total RNA was isolated with PerfectPure RNA Tissue Kit (5 PRIME Inc.). cDNA was synthesized by iScript cDNA Synthesis Kit (BIO-RAD), using 1µg of total RNA and oligodT primers (Table S1). cDNA was evaluated with qRT-PCR (StepOne Plus, Applied Biosystems), and mRNA was normalized to Gapdh, as previously described (Al-Share et al., 2015).

Statistical analysis

Data were analyzed using one-factor ANOVA analysis with Bonferroni correction or Student’s t-test. P<0.05 were statistically significant.
Results

Age-dependent changes in body composition of Cc2−/− males

Consistent with previous reports (Heinrich et al., 2010, Ghanem et al., 2016, Patel et al., 2012), 5-month-old Cc2−/− males display comparable body weight to their Cc2+/+ wild-type counterparts (Table 1). This is accompanied by elevated lean mass with a reciprocal reduction in fat mass, as assessed by NMR analysis (Table 1). Starting at 9 months of age; however, male mutants begin to gain more body weight (Table 1), with a significant increase in fat mass and a reciprocal reduction in lean mass by comparison to age-matched wild-types (Table 1).

Age-dependent changes in insulin sensitivity and glucose tolerance of Cc2−/− males

As expected from hyperinsulinemic-euglycemic clamp analysis (Ghanem et al., 2016, Patel et al., 2012), 5-month-old Cc2−/− males exhibit stronger glucose and insulin tolerance than Cc2+/+ (Fig. 1A). However, starting at 9 months, they begin to show intolerance to exogenous insulin (Fig. 1B) and glucose [both intraperitoneally (Fig. 1B) and orally-administered (Fig. S1)]. They also start exhibiting elevated fed, but not fasting glucose levels by comparison to Cc2+/+ mice with a rise in plasma NEFA (Table 1).

Consistent with the emergence of insulin resistance at this age, insulin excursion by ~2-4 fold at 7hrs of refeeding (RF) following an overnight fast (F) induces phosphorylation of the insulin receptor-β-subunit (IRβ), Akt in the liver (i) and skeletal muscle (ii) of 5- but not 9-month-old Cc2−/− mice, as demonstrated by sequential immunoblotting (Ib) with antibodies against phosphorylated antibodies (a-pIRβ and a-pAkt) followed by reimmunoblotting (reIb) with antibodies against these proteins (Fig.
Similarly, CEACAM1 phosphorylation in response to insulin is blunted in mutant mice livers starting at 9 months (Fig. 2Ai).

**Hyperphagia drives insulin resistance in older Cc2−/− males**

Cc2−/− mice exhibit a significant increase in daily food consumption at all ages (Fig. 2Bi, only 9-month-old mice are shown). Subjecting them to a pair-feeding (PF) regimen for 2 weeks to restrict their food intake and reduce their body mass to a level comparable to *ad libitum*-fed Cc2+/+ mice (AL-Cc2+/+) (Fig. 2Bii) restores their tolerance to exogenous insulin (Fig. 2Biii, PF-Cc2−/− vs AL-Cc2+/+). This demonstrates that insulin resistance in older Cc2−/− males is primarily caused by hyperphagia.

**Decreased energy expenditure in older Cc2−/− males**

Indirect calorimetry analysis (Fig. 3) revealed that Cc2−/− mice exhibit higher energy expenditure (EE) and VO₂ consumption than age-matched Cc2+/+ males in both light and dark cycles (shaded) at 5 months of age, as previously shown (Patel et al., 2012). Spontaneous physical activity is also elevated in the 5-month-old mutants. However, starting at about 8 months, mutant mice begin to exhibit a decrease in EE and VO₂ consumption without a significant change in locomotor activity until about 12 months of age at which point, their physical activity in the dark cycle declines to become lower than in wild-types (Fig. 3).

Consistent with the skeletal muscle being a major site of energy expenditure, mRNA levels of genes involved in fatty acid transport (Cd36, Fatp1) and b-oxidation (Ppara, Cpt1b, Cox2, Ucp3) are higher in the 5-month-old mutants than wild-types, but
are lower at 12 months (Table 2). Consistently, skeletal muscle’s triacylglycerol (TG) content is higher in 12-month-old mutants relative to wild-types (Table 1). This could be in part, related to a higher sympathetic nervous outflow to skeletal muscle at 5 months of age that became lower at 12 months, as suggested by the mRNA levels of the surrogate marker, the adrenergic-b-receptor 2 (Adb2r) (Table 2) (Onai et al., 1995, Shiuchi et al., 2009).

We have shown that 5-month-old Cc2⁻/⁻ males exhibit an increase in sympathetic tone to white adipose tissue (Patel et al., 2012). Consistently, mRNA levels of Ucp1 and the adrenergic-b-receptors 2 and 3 (Adb2/3r), and of type 2 deiodinase (Dio2), a key gene in brown adipose tissue (de Jesus et al., 2001), are elevated in gonadal white adipose tissue (WAT) from 5-, but not 12-month-old mutants (Table 2). This agrees with insulin sensitivity, the overall lean phenotype and the higher basal metabolic rate in 5-, but not 12-month-old Cc2⁻/⁻ males.

**Sustained enhanced insulin secretion in aged Cc2⁻/⁻ males**

*Cc2⁻/⁻* males manifest higher basal and oral glucose-induced GLP-1 levels than age-matched wild-type mice at 5 months of age (Ghanem et al., 2016). As Table 1 shows, plasma GLP-1 remains elevated in mutant mice up to 12 months of age. Throughout all age groups, they acutely release more insulin in response to an intraperitoneal injection of glucose relative to wild-type *Cc2⁺/⁺* mice (Fig. 4A). The slight rise in insulin excursion at 120min post-injection beginning at 9 months is consistent with the development of insulin resistance at this age. Of note, the increase in insulin secretory function occurs
without any significant change in either a- or b-islet cell area relative to total pancreas in $Cc2^{-/-}$ mice even at 12 months of age (Fig. 4B).

**More robust age-dependent progressive decrease of hepatic CEACAM1 level in older $Cc2^{-/-}$ males**

Despite the rise in insulin secretion, plasma insulin level is normal in 5-month-old mice, consistent with insulin sensitivity at this age (Patel et al., 2012). This is likely due to the ~2-fold increase in hepatic insulin clearance, as assessed by steady-state C-peptide/insulin molar ratio (Fig. 5A). At 9 months, chronic hyperinsulinemia begins to develop in male mutants, in parallel to the gradual reduction in insulin clearance (Fig. 5A). Mechanistically, the age-related decline in hepatic insulin clearance is likely due to changes in hepatic content of CEACAM1 (Fig. 5B), which upon its phosphorylation by the receptor promotes receptor-mediated insulin uptake by the hepatocyte to be targeted for degradation (Poy et al., 2002, DeAngelis et al., 2008). Mice with liver-specific inactivation or with null deletion of Ceacam1 manifest chronic hyperinsulinemia resulting from impaired insulin clearance (Poy et al., 2002, DeAngelis et al., 2008). Of note, hepatic Ceacam1 mRNA levels appears to progressively decline with age in both genotypes, reaching 25%-50% the original level at 12 months of age (Fig. 5B).

The reduction of hepatic Ceacam1 levels in $Cc2^{-/-}$ livers at an older age is likely due to blunted insulin-induced transcription of Ceacam1 under conditions of insulin resistance (Fig. 5B) (Najjar et al., 1996). It can also be due to the rise in hepatic Ppara mRNA level (Table 2) and its activation by elevated plasma NEFA (Table 1) and their
uptake into the hepatocyte via CD36 (Table 2) (Ramakrishnan et al., 2016), as suggested by the increase in the mRNA levels of its transcriptional targets (Cpt1 and Cd36) in 12-month-old mutant mice (Table 2). The decrease in hepatic Ceacam1 level (and phosphorylation-Fig. 2A) and increase in the expression of genes involved in fatty acid b-oxidation (Ppara, Cpt1) is consistent with restricted lipid accumulation in the liver of older mutants (Table 1) (Ramakrishnan et al., 2016).

Insulin suppresses hepatic glucose production by inhibiting gluconeogenesis and stimulating net hepatic glucose uptake and subsequent glycogen synthesis (DeFronzo 1998). Higher fasting Pepck mRNA levels in 12-month-old mice points to increased gluconeogenesis. However, this does not translate into fasting hyperglycemia (Table 1). Instead, reduced G6Pase mRNA levels and increased hepatic glycogen content in older mice in fasted and refed mice (Fig. 5C) points to targeting G-6-P to the glycogen synthetic pathways in older Cc2<sup>−/−</sup> mice (Ramakrishnan et al., 2016, Hue et al., 2009).

Discussion

Previous reports showed that Cc2<sup>−/−</sup> males exhibit hyperphagia that fails to translate into obesity and insulin resistance owing to increased energy expenditure. They also develop increased glucose-induced insulin secretion at least partly, via a mechanism depending on GLP-1 release. This study shows that at ~9 months of age, male mutants develop hyperphagia-driven insulin resistance. They also manifest a decline in energy expenditure and physical activity. As summarized in Fig. 6, this energy imbalance occurs together with impaired hepatic insulin clearance that sustains chronic hyperinsulinemia, at least in
part, by failing to offset the persistent increase in insulin secretion.

Whereas hyperphagia occurs in both $C_{c2^{-/}}$ males and females, the age at which its resulting insulin resistance emerges differs between both groups, with female mice developing metabolic derangement at 2 months (Heinrich et al., 2010), and males at $\sim$9 months of age, as the current studies demonstrate. The delay in insulin resistance in males is attributed to the counter-regulatory effect of their early hypermetabolic state that offsets hyperphagia and maintains insulin sensitivity at young age (Patel et al., 2012). This includes complete fatty acid b-oxidation in skeletal muscle, a main site of energy expenditure, and increased brown adipogenesis and sympathetic tone to adipose tissue (Patel et al., 2012). In older mice; however, fatty acid b-oxidation in skeletal muscle decreases at $\sim$9 months of age, leading to lipid accumulation and lipotoxicity. As in other murine models of altered metabolism (Koves et al., 2008, Wang et al., 2009), this could contribute to insulin resistance in muscle, as manifested by glucose intolerance and underlined by reduced insulin-stimulated Akt phosphorylation, a key mediator of glucose uptake (Summers et al., 1999). Consistent with limited glucose uptake in skeletal muscle in parallel to age-dependent reduction in metabolically active lean mass and increase in visceral obesity (Yalamanchi et al., 2016), lean mass decreases reciprocally to increased fat mass in older $C_{c2^{-/}}$ males. Given that CEACAM2 is not expressed in skeletal muscle (Han et al., 2001), it is likely that the progressive decrease in energy expenditure in older mutants is caused by altered central regulation of glucose uptake resulting from loss of CEACAM2 in the ventromedial hypothalamus (VMH) (Heinrich et al., 2010), a main central regulator of glucose uptake in skeletal muscle via the sympathetic nervous system (Shiuchi et al., 2009).
Moreover, young male mice exhibit increased sympathetic tone to white adipose tissue, consistent with increased brown adipogenesis in this depot and higher energy dissipation (Patel et al., 2012). Consistently, this study shows that they exhibit increased expression of the surrogate markers of brown adipogenesis (Ucp1 and Dio2) (de Jesus et al., 2001) and activated sympathetic tone (Ucp1, Adb2r and Adb3r) (Onai et al., 1995) in their white adipose tissue. In contrast, these markers are significantly reduced in older mutants. Considering that CEACAM2 is not physiologically detected in adipose tissue (Han et al., 2001), reduced energy dissipation and sympathetic tone in adipose tissues of aged Cc2−/− males is likely mediated by its deletion from brain nuclei participating in the neuroendocrine regulation of energy balance, such as VMH (Saito et al., 1985, King et al., 2006).

Insulin resistance is heralded by chronic hyperinsulinemia. Ceacam2 deletion causes an increase in insulin secretion and an amplified acute excursion of insulin in response to glucose across all age groups in male mutants. Elevated insulin secretion is in part mediated by increased release of GLP-1 (Ghanem et al., 2016), an incretin that potentiates glucose-stimulated insulin secretion from pancreatic b-cells (D'Alessio et al., 1994, Reimann et al., 2002, Habib et al., 2013). This is supported by persistently higher plasma GLP-1 levels and an intact b-cell area at 12 months of age.

Despite the increase in insulin secretion caused by Ceacam2 deletion at an early age, chronic hyperinsulinemia does not develop in mutant males until ~9 months. At an earlier age, elevated insulin secretion is countered by an increase in hepatic insulin clearance as shown by the ~2-fold higher steady-state plasma C-peptide/insulin molar ratio in the young mutants. This is likely mediated by insulin’s induction of hepatic
CEACAM1 level resulting from increased transcriptional activity of Ceacam1 promoter (Najjar et al., 1996) and its mRNA and protein levels (Ramakrishnan et al., 2016). At 9 months of age; however, insulin clearance begins to decline substantially, likely mediated by a progressive decrease in hepatic CEACAM1 expression. Whereas this remarkable decrease in Ceacam1 mRNA could stem from a compromised ability of insulin to induce Ceacam1 transcription under conditions of hyperphagia-driven insulin resistance, it can also stem from the activation of Pparα by fatty acids that are mobilized from visceral adipose tissue (Ramakrishnan et al., 2016). The consequence of the decrease in hepatic CEACAM1 level is to provide a positive feedback mechanism on fatty acid b-oxidation (Ramakrishnan et al., 2016), a mechanism that would prevent lipid accumulation in aged Cc2−/− livers. Additionally, increase in fatty acid b-oxidation produces acetyl-CoA that inhibits pyruvate dehydrogenase to prevent glycolysis and reroute pyruvate to gluconeogenesis (supported by increased Pepck mRNA levels). This also causes cytoplasmic citrate to inhibit 6-phosphofructo-1-kinase, increasing G-6-P production. With reduced G6Pase mRNA levels, G-6-P is rerouted to the glycogen synthetic pathways (Salaheldeen et al., 2012, Hue et al., 2009). This is consistent with a role for CEACAM1 in hepatic gluconeogenesis via regulating b-oxidation of lipolysis-derived fatty acids (Titchenell et al., 2016). This limits fasting hyperglycemia in parallel to increased insulin secretion.

Whereas age-related hyperinsulinemia, insulin resistance and visceral obesity (Fink et al., 1983, Short et al., 2003, Oya et al., 2014) are commonly associated with reduced glucose uptake in skeletal muscle in parallel to reduced metabolically active lean mass (Yalamanchi et al., 2016), decreased fenestration in the liver sinusoidal endothelium
and other structural changes causing reduction in insulin uptake into the hepatocyte to undergo clearance has recently emerged as a mechanism underlying age-related hyperinsulinemia and insulin resistance (Mohamad et al., 2016). The current studies provide an alternative mechanism of hyperinsulinemia in older $Cc2^{−/−}$ male mutants implicating a progressive reduction in hepatic CEACAM1-mediated insulin clearance that fails to counter persistent GLP-1–mediated release of insulin in older mutant mice. This points to compromised hepatic insulin clearance as a major contributor to the chronic hyperinsulinemic state that develops in older $Cc2^{−/−}$ male mice, in agreement with several clinical studies showing reduction in insulin clearance as a critical player in the pathogenesis of metabolic abnormalities associated with obesity, glucose intolerance and insulin resistance (Valera Mora et al., 2003, Lee et al., 2013, Marini et al., 2014).

More studies are needed to delineate the role of the decline in hepatic CEACAM1 levels with age, but the current studies are the first to raise the possibility of a mechanistic underpinning involving altered hepatic insulin clearance in age-related insulin resistance (Fig. 6). More specifically, the data propose that loss of CEACAM2 from critical brain centers involved in the regulation of food intake causes hyperphagia, followed by insulin resistance (including lipolysis). This lowers hepatic CEACAM1 level and phosphorylation to impair hepatic insulin clearance and cause an unmatched increase in insulin secretion, thus leading to chronic hyperinsulinemia and sustained insulin resistance with age.
Acknowledgements The authors thank M. Kopfman at the Najjar Laboratory for her technical assistance in the generation and maintenance of mice, and in carrying out routine DNA and RNA analyses.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contributions S.M. Ghanem researched data, designed experiments wrote a first draft and revised the manuscript. H.T. Muturi, A.M. DeAngelis, J. Hu and G. Heinrich researched data. G. Heinrich was involved in data analysis and discussions. He also critically read the manuscript. R.N. Kulkarni supervised the experiments pertaining to pancreatic cell areas and analyzed data. S.M.N. was responsible for study design, conceptualization, data analysis and results interpretation, and reviewing the manuscript. S.M.N. had full access to all the data of the study and takes responsibility for the integrity and accuracy of data analysis and the decision to submit and publish the manuscript. All authors have given final approval of this version to be published.
**Figure legends**

**Fig. 1** Male mice (5-12 months of age, \( n \geq 5 \)/genotype/age group) were subjected to intraperitoneal injections of glucose (1.5g/kg BW) or insulin (0.75U/kg BW) to assess blood glucose levels at 0-120 and 0-180min post-injection, respectively. Area under the curve (AUC) was measured and represented in bar graphs. Values are expressed as means±SEM. *\( P < 0.05 \) in \( Cc2^{-/-} \) (black bars or squares) vs \( Cc2^{+/+} \) mice (white bars or circles).

**Fig. 2** A. Phosphorylation (and activation) of the Insulin receptor-β-subunit (IR\( \beta \)), Akt and CEACAM1 in response to insulin excursion during refeeding (RF) for 7hrs following an overnight fast (F) was assessed by immunoblotting (Ib) proteins using antibodies against phosphorylated proteins (a-pIR\( \beta \), a-pAkt and a-pCEACAM1) in lysates from livers and skeletal muscle of mutants at ages 5, 9 and 12 months. Reimmunoblotting (reIb) with antibodies against IR\( \beta \), Akt and CEACAM1 was performed to normalize against the amount of loaded proteins. Each set is a representative of 3 independent experiments performed on different mice. B. Daily food consumption over 5 consecutive days was assessed in 9-month-old mice (\( n = 5 \)/genotype) to determine hyperphagia in mutant mice (i). Some mutants were subjected to a pair-feeding (PF) regimen for 2 weeks to decrease their body weight to the level of \( ad libitum \)-fed \( Cc2^{+/+} \) mice (AL\(-Cc2^{+/+}\)). At the end of the feeding period, body mass (ii) and insulin tolerance (iii) were assessed. Values are expressed as mean±SEM at each time point. *\( P < 0.05 \) vs AL\(-Cc2^{+/+}\).
Fig. 3  

$Cc2^{+/+}$ (white bars) and $Cc2^{-/-}$ (black bars) mice ($n=5$/genotype) were individually caged, given free access to food and subjected to indirect calorimetry to analyze energy expenditure (EE), VO$_2$ consumption (A-B, D-E, G-H), and spontaneous locomotor activity (C, F, I). Measurements over the 24hr-period (starting at 600h) were collected. Graphs show average hourly data over each 12hr-period of the light (600-1800h) and dark (1800-600h) cycles (shaded) during the last 3 consecutive days. Values are expressed as means±SEM. *$P<0.05$ in $Cc2^{-/-}$ (dark bars) vs $Cc2^{+/+}$ mice (white bars).

Fig. 4  A. Glucose-mediated insulin levels were measured at 0-120min post-intraperitoneal injection (IP) of age-matched 5, 9, and 12-month-old mice ($n\geq5$/genotype/age group). Values are expressed as means±SEM. *$P<0.05$ in $Cc2^{-/-}$ (black bars or squares) vs $Cc2^{+/+}$ mice (white bars or circles). B. Pancreas sections from 5 of each of the 12-month-old $Cc2^{+/+}$ and $Cc2^{-/-}$ mice were fixed and immunostained with antibodies against insulin (red) and glucagon (green). α (i) and β (ii) cell areas were estimated by morphometric analysis of islets from $Cc2^{+/+}$ and $Cc2^{-/-}$ mice and calculated relative to pancreas area. Values, expressed as means±SEM in arbitrary units (AU), are presented in the bar graphs below.

Fig. 5  A. Steady-state plasma insulin and C-peptide levels were determined in triplicate from frozen plasma derived from $\geq6$ mice/genotype/age group to calculate the C-peptide/Insulin molar ratio as a surrogate measure of insulin clearance. B. Ceacam1 mRNA content was analyzed in triplicate in frozen liver tissues from mice aged 2-12 months ($\geq5$ mice/genotype/age group). C. Glycogen content was assayed in triplicate in
frozen livers from \( \geq 5 \) mice/genotype/age group. Values from A-C are expressed as means±SEM. \(*P<0.05\) \( \text{Cc2}^{-/-} \) (black bars) vs \( \text{Cc2}^{+/+} \) (white bars)/age group. \( \dagger P<0.05\) vs mice at the earliest age examined.

**Fig. 6.** Metabolic regulations in 5-month-old (left panel) and 9 month-old (right panel) \( \text{Cc2}^{-/-} \) males. SNA, sympathetic nervous activity; EE, energy expenditure; FAO, fatty acid b-oxidation; TG, triacylglycerol; FFA, free fatty acids; plus sign, positive feedback mechanism.

**Fig. S1.** Male mice (5 to 12 months of age, \( n=5 \)/genotype/age group) were subjected to an oral gavage of glucose (3 g/kg BW) to assess blood glucose levels at 0-120 min post-injection. Area under the curve (AUC) was measured and represented as a bar graph. Values are expressed as means±SEM. \(*P<0.05\) in \( \text{Cc2}^{-/-} \) (black bars or squares) vs \( \text{Cc2}^{+/+} \) mice (white bars or circles).
References


Bingham NC, Anderson KK, Reuter AL, Stallings NR, Parker KL (2008) Selective loss of leptin receptors in the ventromedial hypothalamic nucleus results in increased adiposity and a metabolic syndrome. Endocrinology 149: 2138-2148


King BM (2006) The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight. Physiol Behav 87: 221-244


Table 1  Plasma and tissue biochemistry in male mice.

<table>
<thead>
<tr>
<th></th>
<th>5 Months</th>
<th>9 Months</th>
<th>12 Months</th>
<th>5 Months</th>
<th>9 Months</th>
<th>12 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cc2(+/+)</td>
<td>Cc2(-/-)</td>
<td>Cc2(+/+)</td>
<td>Cc2(-/-)</td>
<td>Cc2(+/+)</td>
<td>Cc1(-/-)</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>28.7 ± 0.3</td>
<td>29.9 ± 0.4</td>
<td>30.9 ± 0.2</td>
<td>40.6 ± 2.8*</td>
<td>33.7 ± 0.7</td>
<td>44.2 ± 5.0*</td>
</tr>
<tr>
<td>% Fat Mass</td>
<td>9.2 ± 0.8</td>
<td>9.0 ± 0.1*</td>
<td>11.5 ± 0.6</td>
<td>18.3 ± 2.1*</td>
<td>12.4 ± 0.1</td>
<td>22.1 ± 0.2*</td>
</tr>
<tr>
<td>% Lean Mass</td>
<td>64.7 ± 0.6</td>
<td>73.2 ± 0.7*</td>
<td>63.7 ± 0.5</td>
<td>58.3 ± 1.8*</td>
<td>62.2 ± 0.1</td>
<td>56.0 ± 0.2*</td>
</tr>
<tr>
<td>Fasting Glucose (mg/dl)</td>
<td>87. ± 2.</td>
<td>81. ± 6.</td>
<td>84. ± 3.</td>
<td>92. ± 6.</td>
<td>85. ± 3.</td>
<td>92. ± 8.</td>
</tr>
<tr>
<td>Fed Glucose (mg/dl)</td>
<td>168. ± 4.</td>
<td>156. ± 5.</td>
<td>162. ± 6.</td>
<td>183. ± 4.</td>
<td>164. ± 6.</td>
<td>187. ± 8.</td>
</tr>
<tr>
<td>Plasma NEFA (mEq/l)</td>
<td>0.38 ± 0.02</td>
<td>0.22 ± 0.01*</td>
<td>NA</td>
<td>NA</td>
<td>0.38 ± 0.03</td>
<td>0.53 ± 0.01†</td>
</tr>
<tr>
<td>Plasma TG (mg/dl)</td>
<td>45.3 ± 0.7</td>
<td>31.0 ± 1.3*</td>
<td>NA</td>
<td>NA</td>
<td>48.1 ± 0.6</td>
<td>66.6 ± 1.2†</td>
</tr>
<tr>
<td>Sk. Muscle TG (μg/mg)</td>
<td>4.5 ± 0.1</td>
<td>2.8 ± 0.1*</td>
<td>NA</td>
<td>NA</td>
<td>4.7 ± 0.1</td>
<td>6.6 ± 0.1*†</td>
</tr>
<tr>
<td>Hepatic TG (μg/mg)</td>
<td>56.4 ± 0.6</td>
<td>55.8 ± 0.7</td>
<td>NA</td>
<td>NA</td>
<td>57.5 ± 0.8</td>
<td>55.6 ± 0.9</td>
</tr>
<tr>
<td>Plasma GLP-1 (pM)</td>
<td>1.2 ± 0.1</td>
<td>4.2 ± 0.8*</td>
<td>1.3 ± 0.2</td>
<td>4.0 ± 0.9*</td>
<td>0.9 ± 0.2</td>
<td>4.6 ± 0.7*</td>
</tr>
</tbody>
</table>

Male mice (n=5-6/genotype) were fasted overnight before retro-venous blood was drawn for blood drawn. Values are expressed as mean±SEM. *\(P<0.05\) versus Cc2\(+/+\)/age group; †\(P<0.05\) versus 5-month-oldCc2\(-/-\) mice. NEFA, Non-esterified fatty acids. TG, triacylglycerol. Sk. Muscle, skeletal muscle.
Table 2  Quantitative real-time PCR analysis of mRNA levels of genes involved in fat metabolism, brown adipogenesis and gluconeogenesis.

<table>
<thead>
<tr>
<th></th>
<th>5 Months</th>
<th>12 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cc2+/+</td>
<td>Cc2−/−</td>
</tr>
<tr>
<td><strong>Skeletal Muscle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd36</td>
<td>0.52 ± 0.04</td>
<td>1.16 ± 0.05*</td>
</tr>
<tr>
<td>Fatp1</td>
<td>0.72 ± 0.05</td>
<td>1.79 ± 0.10*</td>
</tr>
<tr>
<td>Pparα</td>
<td>0.55 ± 0.03</td>
<td>1.40 ± 0.08*</td>
</tr>
<tr>
<td>Cpt1b</td>
<td>0.76 ± 0.01</td>
<td>1.07 ± 0.03*</td>
</tr>
<tr>
<td>Cox2</td>
<td>0.62 ± 0.02</td>
<td>1.23 ± 0.02*</td>
</tr>
<tr>
<td>Ucp3</td>
<td>0.58 ± 0.03</td>
<td>1.30 ± 0.02*</td>
</tr>
<tr>
<td>Pdk4</td>
<td>0.56 ± 0.01</td>
<td>1.03 ± 0.05*</td>
</tr>
<tr>
<td>Adβ2r</td>
<td>1.27 ± 0.04</td>
<td>2.73 ± 0.14*</td>
</tr>
<tr>
<td><strong>WAT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ucp1</td>
<td>1.70 ± 0.09</td>
<td>3.95 ± 0.09*</td>
</tr>
<tr>
<td>Dio2</td>
<td>1.46 ± 0.08</td>
<td>6.10 ± 0.12*</td>
</tr>
<tr>
<td>Adβ3r</td>
<td>1.01 ± 0.06</td>
<td>3.19 ± 0.19*</td>
</tr>
<tr>
<td>Adβ2r</td>
<td>1.15 ± 0.05</td>
<td>2.17 ± 0.10*</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd36</td>
<td>1.24 ± 0.03</td>
<td>1.35 ± 0.03</td>
</tr>
<tr>
<td>Fasn</td>
<td>0.33 ± 0.02</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Pparα</td>
<td>1.27 ± 0.03</td>
<td>1.16 ± 0.02</td>
</tr>
<tr>
<td>Cpt1b</td>
<td>0.94 ± 0.03</td>
<td>1.05 ± 0.05</td>
</tr>
<tr>
<td>Pepck</td>
<td>0.54 ± 0.05</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>G6Pase</td>
<td>0.58 ± 0.04</td>
<td>0.68 ± 0.05</td>
</tr>
</tbody>
</table>

Mixed skeletal muscle (gastrocnemius and soleus), white adipose tissue and liver were extracted from male mice (n=5/genotype) at 5 and 12 months of age to determine the mRNA level of genes by qRT-PCR in duplicate. Values are expressed as means ± SEM.  
*P<0.05 versus Cc2+/+/age group. †P<0.05 versus 5-month-old mice/genotype.
### Table S1  Sequence of primers of mouse genes used in Real-time PCR analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence (5’-3’)</th>
<th>Reverse Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCeacam1</td>
<td>AATCTGCCCTGCGCTTGAGGCC</td>
<td>AAATGGACAGTCCCTGAGTACG</td>
</tr>
<tr>
<td>Ucp3</td>
<td>GTCTGCCCTCATCGGCTTGGT</td>
<td>CCTGGCTCCTACCACGTTGGCT</td>
</tr>
<tr>
<td>Cpt1b</td>
<td>AGGCAGAAGAGTGGGGTCTGACT</td>
<td>ACCCTGGCTGCGTAGAAGCTATGT</td>
</tr>
<tr>
<td>Pdk4</td>
<td>CTTTTGGCTGGTTTGGTTA</td>
<td>CCTGGCTTGGGATACCCAGT</td>
</tr>
<tr>
<td>Cox2</td>
<td>ACGAAATCAACAACCCCGTA</td>
<td>GCCAGAAGCACTCGTTATG</td>
</tr>
<tr>
<td>Pparα</td>
<td>TCACAAGTGCCGTGCTGTGTCG</td>
<td>CAGGTAAGGCTGCTGGATTC</td>
</tr>
<tr>
<td>Cpt1</td>
<td>AGGCAGAAGAGTGGGGTCTGACT</td>
<td>ACCCTGGCTGCGTAGAAGCTATGT</td>
</tr>
<tr>
<td>Cd36</td>
<td>TCTTGGGTACAGCAAGGCGACATA</td>
<td>AGCTATGACAGCATGGAACATGACG</td>
</tr>
<tr>
<td>Ucp1</td>
<td>GGCGCCCTTGTAACACAAA</td>
<td>GTCGGTCTTTCTTCTGGTTA</td>
</tr>
<tr>
<td>Dio2</td>
<td>AAATGACCCCCCTTTGTTTCC</td>
<td>TTCCCATTATCCTTTC</td>
</tr>
<tr>
<td>G6Pase</td>
<td>TCCGGGAGAAGACACAGAG</td>
<td>CAACCTTAATATACGCAAATG</td>
</tr>
<tr>
<td>Pepck</td>
<td>CTTCCTGCGCAAGGCGTACCC</td>
<td>TTGGGATGGGCAC</td>
</tr>
<tr>
<td>Fasn</td>
<td>ACTGTGAAAGCATGCTCCCTGGA</td>
<td>AAGCAACCTCCACTCTCGTTA</td>
</tr>
<tr>
<td>Fatp1</td>
<td>GCAGAAAGCGCAGGAAGA</td>
<td>GGACGTTGGCTGTGATGGG</td>
</tr>
<tr>
<td>Adj3r</td>
<td>GGCACAGGAAAGTGGCACTCCAT</td>
<td>AAGGAAGGGAAAGTGAAGGAAGAC</td>
</tr>
<tr>
<td>Adj2r</td>
<td>ACGAACCCTGGGTGGGTGC</td>
<td>CCAACGCGCACCACCCCTA</td>
</tr>
<tr>
<td>Gapdh</td>
<td>CCAGGTTTGGTCTCTGCGACT</td>
<td>ATACCTGAAATGAGCTTGACAAAGT</td>
</tr>
</tbody>
</table>
**IP Glucose and insulin tolerance tests**

**A. 5 Months**

- Blood glucose (mg/dl)
  - **Cc2**
  - **Cc2**

- Blood Glucose (% Basal)
  - **Cc2**
  - **Cc2**
  - AUC (10^3)

**B. 9 Months**

- Blood glucose (mg/dl)
  - **Cc2**
  - **Cc2**

- Blood Glucose (% Basal)
  - **Cc2**
  - **Cc2**
  - AUC (10^3)

**C. 12 Months**

- Blood glucose (mg/dl)
  - **Cc2**
  - **Cc2**

- Blood Glucose (% Basal)
  - **Cc2**
  - **Cc2**
  - AUC (10^3)

Post-glucose IP (min)

Post-insulin IP (min)
A. Insulin signaling

\textit{i. Liver}

\begin{tabular}{l|ccc|ccc|ccc}
 & \textbf{F} & \textbf{RF} & \textbf{F} & \textbf{RF} & \textbf{F} & \textbf{RF} \\
\hline
\text{lb:}\alpha-\text{pIR}_{\beta} & & & & & & \\
\text{relb:}\alpha-\text{IR}_{\beta} & & & & & & \\
\text{lb:}\alpha-\text{pAkt} & & & & & & \\
\text{relb:}\alpha-\text{Akt} & & & & & & \\
\text{lb:}\alpha-\text{pCC1} & & & & & & \\
\text{relb:}\alpha-\text{mCC1} & & & & & & \\
\end{tabular}

\textit{ii. Sk. Muscle}

\begin{tabular}{l|ccc|ccc|ccc}
 & \textbf{F} & \textbf{RF} & \textbf{F} & \textbf{RF} & \textbf{F} & \textbf{RF} \\
\hline
\text{lb:}\alpha-\text{pIR}_{\beta} & & & & & & \\
\text{relb:}\alpha-\text{IR}_{\beta} & & & & & & \\
\text{lb:}\alpha-\text{pAkt} & & & & & & \\
\text{relb:}\alpha-\text{Akt} & & & & & & \\
\text{lb:}\alpha-\text{pCC1} & & & & & & \\
\text{relb:}\alpha-\text{mCC1} & & & & & & \\
\end{tabular}

B. Pair-feeding at 9 months of age

\begin{tabular}{c|c}
\textit{i.} Daily food intake (g) & \textit{ii.} Body Mass (g) \\
\hline
\text{Cc2}^{-/-} & \text{AL-Cc2}^{-/-} \\
\end{tabular}

\begin{tabular}{c|c}
\textit{iii.} Blood Glucose (\% Basal) & \text{Post-insulin IP (min)} \\
\hline
\text{AL-Cc2}^{-/-} & \text{AL-Cc2}^{+/+} \\
\end{tabular}
Indirect calorimetry

5 Months

A. EE (Kcal/h/kg)

B. VO₂ consumed (mg/h/kg x 10⁻³)

Cc2⁻⁻ Cc2⁻⁻

C. Locomotor Activity (Counts x 10²)

Cc2⁻⁻ Cc2⁻⁻

8 Months

D. EE (Kcal/h/kg)

E. VO₂ consumed (mg/h/kg x 10⁻³)

Cc2⁻⁻ Cc2⁻⁻

F. Locomotor Activity (Counts x 10²)

Cc2⁻⁻ Cc2⁻⁻

12 Months

G. EE (Kcal/h/kg)

H. VO₂ consumed (mg/h/kg x 10⁻³)

Cc2⁻⁻ Cc2⁻⁻

I. Locomotor Activity (Counts x 10²)

Cc2⁻⁻ Cc2⁻⁻
**A. Insulin release in response to glucose**

**5 Months**

![Graph showing insulin release in 5 months for Cc2+/- and Cc2-/- mice.](image)

**9 Months**

![Graph showing insulin release in 9 months for Cc2+/- and Cc2-/- mice.](image)

**12 Months**

![Graph showing insulin release in 12 months for Cc2+/- and Cc2-/- mice.](image)

**B. Area of islet cells in 12-month-old mice**

**Cc2+/-**

![Image of islet cells with area measurements for Cc2+/- mice.](image)

**Cc2-/-**

![Image of islet cells with area measurements for Cc2-/- mice.](image)
Figure 5

A. Hepatic insulin clearance

1. Insulin (pM)

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<th>5</th>
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2. C-peptide (pM)

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3. CII

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B. Age-dependent decrease in Ceacam1 mRNA

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C. Hepatic glycogen content

**Fast**

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**Refeed**

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Figure 6
Supplementary Figure 1

**Oral glucose tolerance test**

![Graph showing oral glucose tolerance test results for 5 and 12 months for Cc2-/- mice. The graphs compare blood glucose levels (mg/dl) over time (min) with AUC (Area Under the Curve) values.](image)
Chapter 5

Role for hepatic CEACAM1 in regulating fatty acid metabolism along the adipocyte-hepatocyte axis

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**Running Title:** Diet-induced Lipolysis and Hepatic Insulin Clearance
Abstract

Carcinoembryonic-related cell adhesion molecule 1 (CEACAM1) regulates insulin sensitivity by promoting hepatic insulin clearance and mediating suppression of fatty acid synthase activity. Feeding C57BL/6J male mice with a high-fat diet for 3-4 weeks triggered a >60% decrease in hepatic CEACAM1 levels to subsequently impair insulin clearance and cause systemic insulin resistance and hepatic steatosis. This study aimed at investigating whether lipolysis drives reduction in hepatic CEACAM1 and whether this constitutes a key mechanism leading to diet-induced metabolic abnormalities. Blocking lipolysis with a daily intraperitoneal injection of nicotinic acid in the last two days of a 30-day high-fat feeding regimen demonstrated that white adipose tissue-derived fatty acids repressed hepatic CEACAM1-dependent regulation of insulin and lipid metabolism in 3-month old male C57BL/6J mice. Adenoviral-mediated CEACAM1 redelivery countered the adverse metabolic effect of the high-fat diet on insulin resistance, hepatic steatosis, visceral obesity and energy expenditure. It also reversed the effect of high-fat diet on inflammation and fibrosis in white adipose tissue and liver. This assigns a causative role for lipolysis-driven decrease in hepatic CEACAM1 level and its regulation of insulin and lipid metabolism in sustaining systemic insulin resistance, hepatic steatosis, and other abnormalities associated with excessive energy supply.

Keywords: CEACAM1; Insulin resistance; Nicotinic acid; Lipolysis; Insulin clearance.
Introduction

Circulating insulin levels, in part determined by hepatic insulin clearance, regulate insulin action (Dankner et al., 2009, Pories et al., 2012, Corkey et al., 2012). Insulin clearance, which occurs mostly in liver and to a lower extent in kidney, but not in skeletal muscle or white adipose tissue (WAT), plays a pivotal role in promoting insulin sensitivity (Ader et al., 2014). If impaired, it contributes to mounting hyperinsulinemia in obese humans (Meistas et al., 1983, Jones et al., 2000), thus constituting a risk factor for metabolic syndrome (Pivovarova et al., 2013, Lee et al., 2013).

Our studies on the carcinoembryonic-antigen related cell adhesion molecule 1 (CEACAM1), a transmembrane glycoprotein that is highly expressed in liver and kidney, but not WAT or skeletal muscle (Najjar 2002), support these findings. Upon its phosphorylation by the insulin receptor, CEACAM1 promotes insulin clearance by upregulating receptor-mediated insulin uptake into clathrin-coated pits and degradation in hepatocytes (Choice et al., 1998). Moreover, it mediates a downregulatory effect on fatty acid synthase (Fasn) activity in response to acute rise in insulin in the first hours of refeeding following an overnight fast (Najjar et al., 2005). This positions CEACAM1 to contribute to the regulation of fatty acid oxidation until glycogen repletion is complete (Ramakrishnan et al., 2016). Mice with null deletion of Ceacam1 (Ce/Cc1–/–) or with liver-specific inactivation of CEACAM1 develop hyperinsulinemia, caused by impaired insulin clearance, followed by insulin resistance, hepatic steatosis and visceral obesity (Poy et al., 2002, Xu et al., 2009, DeAngelis et al., 2008, Lee et al., 2008). Normal acute-
phase insulin release to glucose and intact β-cell function support the observation that chronic hyperinsulinemia is mainly driven by impaired insulin clearance in Cc1^+/– mutants (DeAngelis et al., 2008).

Studies in mice (Al-Share et al., 2015), dogs (Mittelman et al., 2000) and humans (Bakker et al., 2014) demonstrated that defective hepatic insulin clearance is involved in diet-induced insulin resistance. Providing a Western-style diet caused rapid hepatic insulin resistance in healthy young South-Asian men but not Caucasians, in association with altered insulin clearance in the Asian group (Bakker et al., 2014). We have shown that high-fat reduced hepatic CEACAM1 level by >60% and impaired insulin clearance in wild-type mice within 3 weeks to introduce metabolic abnormalities that were prevented by forced transgenic fat-inducible CEACAM1 overexpression in liver (Al-Share et al., 2015). The clinical implication of our studies is underscored by the observed low hepatic CEACAM1 level in obese, insulin-resistant subjects with fatty liver disease (Lee 2011).

Dietary fatty acids can reach the liver via chylomicrons in addition to lipolysis in WAT. In uncomplicated human obesity with low-grade insulin resistance, lipolysis-derived fatty acids are mainly removed by oxidation in the liver (Groop et al., 1991). Lipolysis occurs within few days of the initiation of high-fat intake, owing to dysregulated hypothalamic control, even in the absence of insulin resistance in WAT (Scherer et al., 2012). This could cause rapid hepatic insulin resistance (portal hypothesis) via several mechanisms (Kabir et al., 2005), including PKCδ-mediated pathways (Pereira et al., 2014). With persistent nutritional burden, hepatic lipotoxicity and systemic insulin resistance develop in parallel to the progression of a pro-inflammatory state in WAT.
Release of fatty acids during an acute intralipid infusion reduced hepatic CEACAM1 levels and impaired insulin clearance (Pereira et al., 2014). Thus, we herein investigated the role of lipolysis-derived fatty acids in the suppression of CEACAM1 by high-fat diet, and assessed the significance of this mechanism in diet-induced metabolic abnormalities.

Materials and Methods

Mice maintenance

C57BL/6J mice were kept in a 12-h-dark/light cycle. As reported (Al-Share et al., 2015), male mice (3-month-old) were housed as 3-4 mice/cage and fed ad libitum for 30 days a standard chow (RD) or a high-fat (HF) diet deriving 45:35:20% calories from fat:carbohydrate:protein (Catalog #D12451, Research Diets). On day 28, some HF-fed mice were subjected to a once daily intraperitoneal injection of nicotinic acid (NA) (200 mg/kg BW/day) (Sigma-Aldrich) for 2 days (Girousse et al., 2013, Wanders et al., 2013). The Institutional Animal Care and Utilization Committee approved all procedures.

Metabolic parameters

Mice were overnight-fasted and their retro-orbital venous blood drawn at 1100h the following morning to assess blood, plasma and tissue biochemistry, as previously described (Al-Share et al., 2015).

Insulin and glucose tolerance tests

Mice were fasted for 6h before being injected intraperitoneally (ip) with Human Regular
Insulin (0.75U/kg BW, Novo Nordisk) (for insulin tolerance) or glucose (1.5g/kg body wt of 50% dextrose solution, Dextrose Injection, USP). Glucose was measured in tail blood at 0-180 min post-injection.

**Indirect calorimetry analysis**

Mice were individually caged for 5 days (CLAMS system, Columbus Instrument) and their spontaneous physical activity was determined on the X-(locomotor), Y-(ambulatory), and Z-(standing) axis (Al-Share et al., 2015). Oxygen consumption (VO$_2$), CO$_2$ production (VCO$_2$) and heat production were sampled every 20min and normalized to lean mass. The respiratory exchange rate (RER) was calculated as the VCO$_2$/VO$_2$ ratio. Heat production was calculated as Cv x VO2 normalized to lean body mass; with Cv (Calorific value) being: 3.815+1.232×RER. Data were represented as mean±SEM of light (700h-1900h) and dark (1900h-700h) cycles.

**Fatty acid synthase activity**

As previously described (Najjar 2002), livers were homogenized in buffer containing 20mM Tris (pH 7.5), 1.0mM EDTA, 1.0mM DTT, and phosphatase and protease inhibitors (Najjar et al., 2005). Following centrifugation at 12,500xg for 30 min, 10µl of the supernatant was added to 65µl of the reaction mix. This contains: 166.6µM acetyl-CoA, 100mM potassium phosphate (pH 6.6), 0.1µCi $[^{14}$C] malonyl-CoA (Perkin Elmer, Waltham, MA), and 25nmol malonyl-CoA, in the absence (negative controls) or presence of 500µM NADPH (Sigma Aldrich). 1:1 chloroform:methanol solution was used to stop the reaction. Following centrifugation, the supernatant was vacuum-dried, and the pellet
resuspended in 200µl of water-saturated butanol. To re-extract the upper layer, 200µl of ddH$_2$O was added followed by vortexing, and spinning for 1min. The butanol layer was extracted, dried and counted for the incorporation of radiolabeled malonyl-CoA into palmitate. Fatty acid synthase (Fasn) activity was calculated as cpm of [$^{14}$C] incorporated/µg of cell lysates and the protein concentration was determined by Bio-Rad protein assay.

**Ex-vivo palmitate oxidation**

As previously described (Heinrich et al., 2010) with some modifications, mice were fasted overnight and anesthetized, and the liver removed, weighed, and homogenized in 10mmol/L Tris (pH 7.2), 300mmol/L sucrose, and 2mmol/L EDTA. 1 ml of the homogenate was added to a sealed beaker containing 1ml of solution A and left at 30°C for 45 min. Solution A: 0.2mmol/L of [1-$^{14}$C]palmitate (0.5mCi/mL) (American Radiolabeled Chemicals, Inc) and 2mmol/L ATP in incubation buffer (100mmol/L sucrose, 10mmol/L Tris-HCl, 5mmol/L potassium phosphate, 80mmol/L KCl, 1mmol/L MgCl$_2$, 2mmol/L L-carnitine, 0.1mmol/L malic acid, 0.05mmol/L CoA, 1mmol/L dithiothreitol, 0.2mmol/L EDTA, and 0.5% BSA, pH 7.4). Benzothonium hydroxide (Sigma-Aldrich) was added to a basket attached to the sealed beaker and the reaction was terminated with perchloric acid to recover the radioactive acid soluble metabolites (Hirschey et al., 2010). Trapped CO$_2$ radioactivity and the partial oxidation products were measured by liquid scintillation in CytoCint (MP Biomedicals). The oxidation rate was expressed as the sum of total and partial fatty acid oxidation expressed in nmol/g/min.
Gomori-trichrome staining

Trichrome stain was performed on formalin-fixed adipose tissue using the Thermo Scientific Richard-Allan Scientific Chromaview-advanced Testing (Lester et al., 2015). Adipose tissue was fixed in 10% formalin and replaced by 70% ethanol before undergoing blocking in paraffin. Sections were deparaffinized at 60°C and hydrated in deionized water. Slides were then stained with Bouin’s Fluid at 56°C for 45 min, followed by rinsing in deionized water to remove the yellow color. Slides were placed in Working Weigert’s Iron Hematoxylin at room temperature (RT) for 10 min followed by Trichrome Stain for 15 min, dehydrated sequentially in 1% acetic acid solution for 1 min, 95% ethanol for 30 sec, and 100% ethanol for 1 min (twice). Sections were cleared in Xylene 3 times for 1 min each and mounted.

Immunofluorescence

Whole WAT was formalin-fixed for 24h, transferred to Dulbecco’s PBS (Sigma-Aldrich) at 4°C and permeabilized in 1% TritonX-100/PBS for 15min before staining macrophages with rat anti-mouse F4/80 (Invitrogen) and detecting with donkey anti-rat IgG-conjugated to Alexa-Fluor488. Tissues were incubated with primary antibodies overnight at 4°C then washed 3x with PBS-Tween20 before applying the secondary stain for 2h at room temperature (RT) and washing 3x in PBS-Tween. Stained samples were then counterstained for 25min at RT with 5µM BODIPY-558/568 (Molecular Probes) and Hoechst stain/DAPI (Invitrogen) to visualize lipid and nuclei, respectively. Samples were placed on a coverslip and imaged using a Leica-TCS-SP5 laser-scanning microscope equipped with conventional solid-state and a Ti-sapphire tunable multi-
photon laser (Coherent). Images were acquired in the 3D-XYZ plane in 4μm steps with a 63x objective (NA-0.70) using the sequential scan mode to eliminate any spectral overlap in individual fluorophores. Specifically, Alexa-Fluor488 was excited at 488nm with collection at 500–558nm. The BODIPY-558/568 dye was excited at 561nm and collected at 567–609nm. Selected images are a 2D-representation of the 3D-LSCM-image stack as labeled.

**Western blot analysis**

Proteins were analyzed by SDS-PAGE and immunoprobing with polyclonal antibodies against mouse fatty acid synthase (α-Fasn) (Cell Signaling Technology), phospho-Akt (Ser\textsuperscript{473}) and phospho-Akt2 (p-Akt2) followed by α-Akt or α-Akt2, respectively; custom-made polyclonal antibodies raised in rabbit against mouse CEACAM1 (α-mCC1 Ab3759) (Al-Share et al., 2015), rat CEACAM1 [(αP\textsubscript{3}(488)] and phospho-CEACAM1 (α-pCC1) (Pereira et al., 2014). For normalization, monoclonal antibodies against Actin or GAPDH (Santa Cruz Biotechnology) were used. Blots were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (GE Healthcare Life Sciences Amersham) antibodies, subjected to enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and quantification by densitometry and Image J software (v. 1.40, NIH).

For co-immunoprecipitation, liver lysates (35μg) were immunoprecipitated with α-Fasn antibody, followed by analysis on 5%SDS-PAGE, and sequential immunoblotting with α-mCC1 and α-Fasn antibodies.
Primary hepatocytes isolation and fatty acid treatment

Hepatocytes were isolated by perfusing liver (1ml/min) with Collagenase-Type II (1mg/ml) (Worthington) (Poy et al., 2002). Cells were dispensed in Williams-E complete media (Gibco) containing 10mM lactate, 10nM dexamethasone, 100nM insulin (Sigma Aldrich), 10% FBS and 1% penicillin-streptomycin (Gibco). Cells were plated onto 12-well plates at 2.5x10^5/well and incubated at 37°C in Williams-E medium for 24h before switching to phenol red-free Williams-E medium-supplemented with 10% dialyzed FBS, 1% penicillin-streptomycin for 24h. A fatty acid mixture resembling the dietary fat composition (0.1mM) was added for 24h: 0.035mM palmitate, 0.045mM Oleate, 0.02mM linoleate-2mM insulin-free BSA (Sigma Aldrich) at a 1:5 ratio], supplemented with 10mM lactate.

Luciferase assay

As described (Ramakrishnan et al., 2016), Hepa1-6 mouse-derived cells were seeded at 4.0x10^5 into 6-well plates and at ~ 60-70% confluence, a 24h-transfection was performed with 500ng of promoter constructs containing the wild-type sequence spanning 1100 nucleotides or the ΔPPRE/RXR mutant carrying a mutation between nucleotides –557 and –543 (5'-CAATTCTATGAAATC-3', and 10ng of renilla luciferase (pRL-TK, Promega) using Fugene 6 (Promega). Empty pGL4.10 vector was used as negative control. Cells were then serum-starved, treated with ethanol (Veh) or 0.1mM FA (above) for 24h. Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega).
Adenovirus preparation and cell transduction

The construction of the adenovirus vectors harboring wild-type (WT) and the phosphorylation-defective serine503-to-alanine (S503A) rat CEACAM1 mutant was described (DeAngelis et al., 2008). Adenoviruses were produced in 293AD packaging cells (Agilent Technologies) following LipofectAMINE-mediated transfection (Invitrogen), and concentrated by ultracentrifugation in cesium chloride gradient (Tong et al., 2012). Adenoviral-mediated CEACAM1 overexpression was confirmed by transducing primary mouse hepatocytes prepared from C57BL/6J mice at 1x10^7 particles/well of a 6-well plate for 36h. CEACAM1 level was determined by immunoblotting with anti-rat CEACAM1 antibody (Pereira et al., 2014).

Tail-vein injection of recombinant adenovirus to redeliver CEACAM1 to the liver

2-month-old C57BL/6J mice were HF-fed for 20 days before being subjected to adenoviral infection by tail-vein injection of 1x10^10 particles/mouse and housed individually for 21 days. The control group was injected with Ad-GFP at the same viral titer. Insulin and glucose tolerance were performed 13 and 16 days post-injection, as described below. Mice were subjected to indirect calorimetry before being sacrificed at 21 days post-injection.

Real-time quantitative RT-PCR

Total RNA was isolated with PerfectPure RNA Tissue Kit (5 PRIME Inc.) and cDNA was synthesized by iScript cDNA Synthesis Kit (BIO-RAD), using 1µg of total RNA and oligoT primers (Supplementary Table S1). cDNA was evaluated with qRT-PCR
(StepOne Plus, Applied Biosystems) and mRNA was normalized to 18S or Gapdh, as previously described (Al-Share et al., 2015).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni correction, using GraphPad Prism 6 software. $P<0.05$ was statistically significant.
Results

Nicotinic acid inhibits lipolysis and causes fat accumulation in WAT

To investigate the role of adipocyte-derived fatty acids in diet-induced metabolic abnormalities, mice were HF-fed and treated with NA or saline (S) in the last 2 days of feeding. Relative to regular diet (RD), HF induced body weight, visceral and total fat mass, including brown adipose mass (BAT) with a reciprocal decrease in lean mass (Table 1; HF-S vs. RD-S). Consistently, HF increased fasting plasma non-esterified fatty acids (NEFA) (Table 1) and the mRNA level of hormone sensitive lipase (Hsl) in WAT (12.7 ± 0.56 in HF-S vs 6.77 ± 1.07 in RD-S mice; \( P < 0.05 \)). As expected, NA treatment restored plasma NEFA (Table 1) and Hsl mRNA levels (3.75 ± 0.60 in HF-NA vs 12.7 ± 0.56 in HF-S mice; \( P < 0.05 \)) but maintained the gain in visceral obesity and body weight in response to HF (Table 1). Consistently, plasma leptin levels remained elevated in HF-NA relative to RD-S mice (Table 1).

Histological analysis of H&E stained WAT sections (Fig. 1A) confirmed the maintenance of adipocytes’ expansion to accommodate fat storage in WAT derived from HF-NA mice (adipocyte size of 2078 ± 86 \( \mu m^2 \) in HF-NA vs 1848 ± 91 in HF-S and 576 ± 23 in RD-S).

Nicotinic acid reverses diet-induced fibrosis in WAT

Because visceral obesity is associated with increased fibrosis in WAT in rodents (Yadav et al., 2011, Sun et al., 2014) and humans (Divoux et al., 2010), we then examined whether NA treatment modulates HF-induced fibrosis in WAT. HF induced the mRNA levels of pro-fibrogenic effectors: endotrophin/Col6a3, a protein that promotes metabolic
derangement and fibrosis in adipose tissue (Sun et al., 2014), α-smooth muscle actin (α-Sma), and transforming growth factor-β (Tgf-β) (Supplementary Table S2; HF-S vs RD-S). In contrast, HF lowered the mRNA level of Smad7 that inhibits TGF-β signaling (Nakao et al., 1997, Bitzer et al., 2000) (Supplementary Table S2; HF-S vs RD-S). NA treatment reversed the effect of HF, with a lesser extent in Col6α3 mRNA relative to other markers (Supplementary Table S2). This translated into limiting HF-induction of collagen deposition in WAT by NA, as indicated by Trichrome-C staining (Fig. 1B).

Nicotinic acid induces macrophage inflammation in WAT of HF-fed mice
QRT-PCR analysis in WAT revealed induction of mRNA of macrophages (CD68 and F4/80) and TNFα by HF, but without significantly changing the expression of other pro-inflammatory markers such as IL-1β, IL-6 and interferon γ (INFγ) (Supplementary Table S2). NA further induced the mRNA levels of these markers by ~two-to-threefold. Immunofluorescence analysis of F4/80 (green) in WAT showed evidence of macrophage inflammation in HF-S and HF-NA compared to RD-S mice (Fig. 1C; viii and ix vs vii). In contrast to RD-fed mice (Fig. 1Cx), HF induced macrophage staining around the adipocytes (gray), but to a higher extent in NA-treated vs saline-treated mice (Fig. 1C; xii vs xi), indicating increased macrophage inflammation by NA. Despite fewer macrophages; however, HF-S mice displayed higher plasma NEFA levels than HF-NA (Table 1).

Nicotinic acid reverses diet-induced insulin resistance
Consistent with our previous report (Al-Share et al., 2015), HF caused insulin resistance,
as indicated by fed hyperglycemia (Table 1) and intolerance to exogenous insulin in HF-S vs RD-S [Fig. 2A and accompanying graph of the area under the curve (AUC)]. Both glucose levels (Table 1) and insulin tolerance (Fig. 2A) were restored by NA treatment. To further examine insulin action, we refed mice for 7h after an overnight fast to allow insulin release (Fig. 2Bi). Immunoblotting (Ib) with phosphoAkt antibody (symbol-pAkt) revealed blunted insulin’s ability to phosphorylate Akt at 7h of refeeding (RF) relative to overnight-fasted mice (F) in lysates from liver (Fig. 2Bii), but not WAT (Fig. 2Biii) of HF-fed mice. NA treatment restored insulin-induced Akt phosphorylation in liver (Fig. 2Bii), but blunted it in WAT of HF-Fed mice (Fig. 2Biii).

Nicotinic acid restores insulin clearance in HF-fed mice

Western blot analysis of liver lysates revealed reduction of CEACAM1 (CC1) protein level by ~60% in HF-S vs RD-S mice (Fig. 2Ci). This was restored by NA treatment (Fig. 2Ci; HF-NA vs RD-S). Subsequently, this normalized insulin clearance, assessed by the higher steady-state C-peptide/Insulin molar ratio (Table 1; HF-NA vs HF-S) and lowered plasma insulin levels (Table 1), in parallel to restoring insulin sensitivity (Fig. 2A).

Supporting a direct effect of fatty acids on hepatic CEACAM1 expression, treating primary hepatocytes with 0.1mM of a fatty acid mixture (FA) with a composition resembling that of the dietary fat reduced CEACAM1 protein level (Fig. 2Cii). Consistent with our previous observations (Ramakrishnan et al., 2016), the down-regulatory effect of FA appears to be mediated by Pparα activation, as demonstrated by the ability of FA (0.1mM) to reduce the transcriptional activity of Ceacam1 wild-type promoter (–1100),
but not of the construct harboring a mutation of the sole active Ppar response element (PPRE/RXR) located between nucleotides –557 and –543 (Fig. 2D).

Nicotinic acid restores lipid metabolism in the liver of mice fed a high-fat diet

HF increased fat accumulation in liver, as shown by H&E analysis (Fig. 3A; HF-S vs RD-S) and the high hepatic triacylglycerol content (Table 1). While this could be attributed, at least in part, to reduced fatty acid flux from the adipose tissue to the liver, we investigated whether NA could also affect fatty acid synthesis and β-oxidation in liver. Given the ~3-fold increase in hepatic fatty acid oxidation in HF-S (Fig. 3B; 3.31 ± 0.22 vs 1.07 ± 0.07 nmol/g/min in RD-S; P<0.05), hepatic steatosis could in part, result from elevated fatty acid synthase (Fasn) protein levels (Fig. 3Ci), and activity (Fig. 3Ci) in response to basal hyperinsulinemia (Fig. 2Bi) (Osborne 2000). NA treatment significantly reversed fat accumulation in liver (Fig. 3A; HF-NA vs HF-S) and hepatic triacylglycerol levels (Table 1; HF-NA vs HF-S) in parallel to reversing basal hyperinsulinemia (Fig. 2Bi) and Fasn level (Fig. 3Ci). Consistent with previous report (Najjar et al., 2005), the acute rise of insulin release at refeeding (RF) downregulates Fasn activity relative to fasting (F) in RD-S mice (Fig. 3Ci). This occurred in parallel to induced CEACAM1 phosphorylation as shown by immunoblotting with anti-phospho-CEACAM1 (α-pCC1) antibody (Fig. 3Ci), followed by its increased binding of CEACAM1 to Fasn, as shown by its detection in co-immunoprecipitation (Co-IP) with the Fasn immunopellet from RD-S mice (Fig. 3Ci). In contrast, under chronic hyperinsulinemic conditions and in response to HF, insulin failed to phosphorylate CEACAM1 and induce its binding to Fasn to decrease its activity in HF-S. In HF-fed mice, NA restored refeeding-induced
CEACAM1 phosphorylation (Fig. 3Ci) and its binding to Fasn (Fig. 3Ciii) to downregulate its activity (Fig. 3Ci). Together with maintaining the rise in fatty acid β-oxidation (Fig. 3B; 4.39 ± 0.52 in HF-NA vs 1.07 ± 0.07 nmol/g/min in RD-S; \( P<0.05 \)), NA’s ability to restore the suppressive effect of insulin on Fasn activity could counter the effect of HF diet on fat deposition in liver.

**Hepatic adenoviral-redelivery of CEACAM1 reverses diet-induced metabolic abnormalities**

We then tested whether reduced hepatic CEACAM1 is critical to diet-induced metabolic abnormalities. To this end, we rescued CEACAM1 in the liver using adenoviral-mediated redelivery (Ad) and examined whether this reversed the abnormal diet-induced metabolic phenotype. Mice fed HF for 21 days were injected with comparable titers of Ad-GFP control, wild-type rat CEACAM1 (Ad-rWT), and the S503A phosphorylation-defective CEACAM1 mutant (Ad-rSA). Immunoblotting tissue lysates 3 days post-adenoviral injection with a-mouse CEACAM1 antibody detected endogenous CEACAM1 protein expression in liver, with insignificant expression in WAT and skeletal muscle (Fig. 4Ai; mCC1), as expected from the limited expression of CEACAM1 in these tissues (Najjar 2002). In contrast, immunoblotting with a-rat CEACAM1 antibody revealed restricted expression of rat CEACAM1 (rCC1) to the liver but not WAT or muscle of mice injected with Ad-rWT or Ad-rSA, but not Ad-GFP. Transgenic rat CEACAM1 expression was sustained for at least 21 days, as demonstrated by Western (Fig. 4Aii) and qRT-PCR (Fig. 4Aiii) analyses of liver lysates. Per our earlier report (Al-Share et al., 2015), endogenous mouse Ceacam1 mRNA (Fig. 4Aiii) and CEACAM1 protein (Fig. 4Aii) content were
reduced by >60% by HF. In parallel, HF impaired insulin clearance, as assessed by steady-state C-peptide/insulin molar ratio, and hyperinsulinemia (Table 2; HF-GFP vs RD-GFP). This caused fed hyperglycemia (Table 2), and intolerance to insulin (Fig. 4Bi – blue vs black lanes and AUC bars) and glucose (Fig. 4Bii), as assessed on day 13 and 16 post-injection, respectively. Ad- rWT delivery restored insulin clearance together with plasma insulin and C-peptide levels in HF-fed mice (Table 2; HF-rWT vs RD-GFP). It also restored insulin and glucose tolerance (Fig. 4Bi and 4Bii, respectively; green vs black lanes and AUC bars) and blood glucose levels (Table 2). In contrast, injecting Ad-rSA CEACAM1 mutant (red) failed to restore these metabolic factors (Table 2 and Fig. 4B).

Consistent with the positive effect of hyperinsulinemia on hepatic de novo lipogenesis (Osborne 2000), HF-GFP mice manifested higher mRNA levels of SREBP-1c, the master transcriptional regulator of lipogenic genes, than RD-GFP (Supplementary Table S3). This increased Fasn mRNA (Supplementary Table S3) and protein content (Fig. 5Ai) in the liver, and subsequently, enzymatic activity (Fig. 5Aii).

Additionally, HF induced mRNA levels of genes implicated in fatty acid transport, such as Fatp-1 and Fatp-4 (Supplementary Table S3; HF-GFP vs RD-GFP). In contrast, HF lowered Cpt-1a mRNA levels (Supplementary Table S3). Consistent with the role of CPT-1α in transporting fatty acids to mitochondria for β-oxidation, hepatic mRNA expression (Supplementary Table S3) and plasma levels of FGF21 (Table 2) were ~2-fold lower in HF-GFP than RD-GFP mice. Injecting Ad-rWT, but not Ad-rSA, normalized the level of these genes (Supplementary Table S3) as well as plasma FGF21 in HF-rWT, but not HF-rSA mice (Table 2), further supporting restored fatty acid b-oxidation by Ad-rWT.
redelivery. Together, this yielded limited fat accumulation in the liver of HF-rWT relative to HF-GFP and HF-rSA mice, as shown by histological analysis of the H&E stain of the liver (Fig. 5B), and by hepatic triacylglycerol content (Table 2). Consistent with redistribution of substrates to WAT, adipocytes’ expansion was also restricted in HF-rWT by comparison to HF-GFP mice (Fig. 5C), as opposed to mice injected with Ad-rSA mutant in which adipocytes’ expansion was comparable to that in Ad-GFP controls (Fig. 5C).

Consistent with enhanced inflammation by increased fat accumulation (Najjar et al., 2014), qRT-PCR analysis showed induced pro-inflammatory (F4/80 and Tnf-α), and pro-fibrotic genes (α-Sma and Col6α3) in the liver (Supplementary Table S3) of HF-GFP vs RD-GFP mice; all of which were reversed by Ad-rWT, but not Ad-rSA injection. Of note, qRT-PCR analysis showed comparable mRNA levels of F4/80 and TNF-α in the liver of HF-fed mice 3 days post-injection of Ad-rWT and Ad-rSA CEACAM1 (Supplementary Table S4). This indicates that the changes in the inflammatory signals 21 days post-injection are not related to the acute inflammatory response to adenoviral injection, but rather to the metabolic effect of WT CEACAM1, which appears to require longer than 3 days.

Consistent with the downregulation of Smad-7 expression by TNF-α (Nagarajan et al., 2000), HF reduced the mRNA level of Smad-7 in WAT (Supplementary Table S5; HF-GFP vs RD-GFP). Redelivering Ad-rWT, but not Ad-rSA CEACAM1 to the liver restored Smad-7 levels (Supplementary Table S5), consistent with possible TGF-β inhibition, as manifested by decrease in pro-fibrotic genes, α-Sma and Col6α3, in the WAT of HF-rWT, but not HF-rSA mice (Supplementary Table S5).
Adenoviral-redelivery of CEACAM1 rescues energy expenditure in HF-fed mice

Ad-rWT, but not Ad-rSA CEACAM1 totally reversed body weight gain, visceral adiposity and NEFA plasma levels (Table 2). It also restored the mRNA levels of UCP-1, a marker of brown adipogenesis, in WAT (Supplementary Table S5). Additionally, it reduced, but not fully restored total fat mass (Table 2; HF-rWT vs RD-GFP). This provided impetus to investigate potential changes in energy balance by indirect calorimetry 21 days post-injection. As expected (Al-Share et al., 2015), HF feeding reduced daily food intake in all mouse groups relative to RD-GFP mice (Fig. 6A). Indirect calorimetry analysis over a 24-h period revealed lower energy expenditure (Fig. 6B; heat generation), O₂ consumption (VO₂) (not shown), CO₂ production (VCO₂) (not shown), calculated respiratory exchange ratio (RER) (Fig. 6C), and spontaneous locomotor activity along the combined XYZ axes (Fig. 6D) in HF-GFP relative to RD-GFP mice. Injecting mice with Ad-rWT, but not Ad-rSA CEACAM1, reversed the negative effect of HF on energy expenditure and the spontaneous locomotor activity. Preserved energy expenditure and physical activity could contribute to lower fat mass and body weight in HF-rWT than HF-rGFP and HF-rSA mice (Table 2).
Discussion

High-fat diet causes a progressive decline in hepatic CEACAM1 levels reaching ~60% after three weeks, at which point, it impairs insulin clearance to cause hyperinsulinemia with attendant insulin resistance and hepatic steatosis (Al-Share et al., 2015). Conversely, protecting CEACAM1 by fat-inducible liver-specific CEACAM1 overexpression restricted the metabolic derangement caused by high-fat diet, including visceral obesity and disturbed energy balance (Al-Share et al., 2015). While these studies underscored the importance of CEACAM1-dependent hepatic insulin clearance in promoting insulin sensitivity, they did not fully identify the primary factors that cause diet-induced CEACAM1 repression, or the cause-effect relationship between impaired insulin clearance and insulin resistance in response to high-fat diet. The current studies showed that redistribution of WAT-derived fatty acids to the liver during lipolysis mediated the suppressive effect of high-fat diet on hepatic CEACAM1 expression, and that restoring CEACAM1 function by adenoviral-redelivery completely reversed diet-induced metabolic abnormalities.

Blocking lipolysis with nicotinic acid caused adipocytes’ expansion to accommodate fat retention in WAT. This tissue remodeling was likely facilitated by a parallel decrease in fibrosis, a cellular event commonly found in the white adipose depot of insulin-resistant, obese rodents (Yadav et al., 2011, Sun et al., 2014) and humans (Divoux et al., 2010). The anti-fibrotic effect of nicotinic acid could be mediated by the ~2-fold rise in IFNγ (Bhogal et al., 2005) that could counter the profibrogenic effect of Col6α3 (Sun et al., 2014) and IL-6 (Bhogal et al., 2005), and by the combined effect of the rise in WAT-derived TNFα and plasma leptin levels (Carter-Kent et al., 2008).
While nicotinic acid treatment caused fat accumulation, triggered more inflammation, and blunted insulin signaling in WAT of mice fed a high-fat diet for 30 days, it protected hepatic insulin signaling together with systemic insulin response. In parallel, it protected hepatic CEACAM1 levels against high-fat diet, demonstrating that lipolysis mediated the decline in hepatic CEACAM1 expression. This is consistent with findings of compromised insulin clearance and insulin signaling in parallel to reduced CEACAM1 protein content in rats receiving an intralipid-heparin infusion (Pereira et al., 2014).

Lipolysis could occur shortly after initiating high-fat intake due to dysregulated hypothalamic control in the absence of insulin resistance in WAT (Scherer et al., 2012). Released fatty acids could then suppress hepatic CEACAM1 content, as suggested by their down-regulatory effect on Ceacam1 promoter activity and on its protein level in primary hepatocytes. Acute rise in fatty acids can also activate PKCδ-mediated pathways to impair insulin signaling in liver (Pereira et al., 2014), which would in turn, suppress insulin-mediated Ceacam1 transcription (Najjar et al., 1996). We have shown that repression of CEACAM1 does not translate into systemic insulin resistance until about 3 weeks of high-fat feeding (Park et al., 2005) when hepatic CEACAM1 content is reduced by >60% (Al-Share et al., 2015). Complete reversal of these metabolic abnormalities by rescuing wild-type CEACAM1 in the liver of mice kept on a high-fat diet demonstrates that the decrease in hepatic CEACAM1 plays a causative role in sustaining systemic insulin resistance and hepatic steatosis in response to high-fat diet.

The physiological consequence of reduced CEACAM1 expression by high-fat diet appears to maintain hepatic fatty acids β-oxidation at times of excessive energy supply. We have reported that CEACAM1 down-regulates Fasn activity to prevent steatosis in
liver (Najjar et al., 2005). We have also shown that PPARα reduces Ceacam1 transcription to regulate fatty acid β-oxidation during the fasting-refeeding transition (Ramakrishnan et al., 2016) and in response to fenofibrate treatment (Ramakrishnan et al., 2016). Because fatty acids are the endogenous ligands of PPARα (Bays et al., 2004), it is conceivable that adipose tissue-derived fatty acids reduce CEACAM1 levels by a PPARα-mediated mechanism as shown by failure of fatty acids to downregulate the transcriptional activity of a promoter construct bearing a mutation on the active PPRE/RXR site in Ceacam1 promoter, as they did to the wild-type promoter. As recently shown (Ramakrishnan et al., 2016), suppressing CEACAM1 would provide a positive feedback mechanism on β-oxidation as it is expected to alleviate the negative effect of CEACAM1 on Fasn activity (Najjar et al., 2005) and subsequently, reduce malonyl-CoA level to relieve its inhibitory effect on fatty acids translocation to the mitochondria to undergo β-oxidation. This presents a novel mechanistic underpinning for the regulation of lipid oxidation by plasma fatty acids (Groop et al., 1991).

Adenoviral-redelivery of wild-type CEACAM1 in the liver restored insulin clearance and subsequently, insulin sensitivity and normal lipid metabolism. It also restored locomotor activity and energy expenditure in mice fed a high-fat diet in parallel to reversing the gain in body weight and visceral adiposity. Given that CEACAM1 is not produced by adipose tissue (Najjar 2002), it is likely that adenoviral delivery of CEACAM1 drives the expression of a set of factors that mediate this positive effect on energy expenditure and adipose tissue biology (reversal of adipocytes’ expansion and limited fibrosis and inflammation). One of these factors might be the rise in plasma FGF21 (Emanuelli et al., 2014, Owen et al., 2014), which induces locomotor activity (Cornu et al., 2014) to elevate
energy dissipation (Rosenbaum et al., 1998, Choi et al., 2015). Recapitulating the protective effect of forced expression of hepatic wild-type CEACAM1 on metabolism (Al-Share et al., 2015) and on adipose tissue biology and energy expenditure (Lester et al., 2015) in response to high-fat diet, the restorative metabolic effect caused by hepatic adenoviral re-delivery of wild-type CEACAM1 on adipocytes supports the critical role of hepatocytic CEACAM1 in regulating insulin action and metabolism in other tissues. Contrary to wild-type CEACAM1, adenoviral-delivery of the Ad-rSA phosphorylation-defective CEACAM1 mutant failed to reverse the negative metabolic effects of high-fat diet. Using the L-SACC1 transgenic mouse with liver-specific dominant-negative overexpression of this SA phosphorylation-defective mutant, we demonstrated that impairment of insulin clearance causes chronic hyperinsulinemia followed by systemic insulin resistance and increased lipid production in liver and redistribution to adipose tissue to cause visceral obesity with increased lipolysis and FFA output (Poy et al., 2002). Consistent with downregulated insulin receptor by chronically elevated insulin levels (Cook et al., 2015, Shanik et al., 2008), L-SACC1 mice manifested reduced insulin receptor number and compromised insulin-induced signaling in hepatocytes (Poy et al., 2002) in addition to reduced ability of insulin to suppress hepatic glucose production, as demonstrated by hyperinsulinemic-euglycemic (Park et al., 2006). Additionally, L-SACC1 mice developed insulin resistance including reduced glucose transport in muscle and adipose, and increased lipid accumulation in liver and peripheral tissues (Park et al., 2006). Hyperinsulinemia can cause insulin resistance in adipose tissue by reducing Glut4-mediated glucose transport (Gonzalez et al., 2011). Moreover, chronic hyperinsulinemia can cause insulin resistance to the suppression of plasma FFA levels and increasing de
novo lipogenesis but not with regard to hepatic gluconeogenesis (Koopmans et al., 1999). Hepatic insulin resistance and increased gluconeogenesis appear to be regulated by increased mobilization of FFA and adipokines from white adipose tissue (Perry et al., 2015, Titchenell et al., 2016). Consistently, inhibiting lipolysis and inducing fatty acid oxidation restored insulin action in L-SACC1 transgenics (Dai et al., 2004), pointing to the role of altered fat metabolism in their sustained insulin resistance that was secondary to impaired insulin clearance. Given that high-fat diet increases Apolipoprotein A1 (Al-Share et al., 2015), it is conceivable that elevated plasma FFA in L-SACC1 mice maintain the elevated level of the dominant-negative rat transgene expression driven by Apolipoprotein A1 promoter, while repressing that of the mouse endogenous gene by a PPARα-dependent mechanism (Ramakrishnan et al., 2016). This could contribute to the suppression of endogenous CEACAM1 activity on hepatic insulin clearance and lipid metabolism by the S503A phosphorylation-defective Ceacam1 mutant transgene.

In summary, the current studies provide a novel role for hepatic CEACAM1-dependent pathways in regulating fatty acid metabolism along the adipocyte-hepatocyte axis in response to excess energy intake. Given that hepatic CEACAM1 content is markedly reduced in the liver of insulin-resistant obese subjects with fatty liver disease (Lee 2011), the current findings propose that inducing CEACAM1 could constitute a critical therapeutic target that serves to mitigate diet-induced metabolic abnormalities, including obesity and fatty liver disease.
Author Contributions

L.R. researched data and designed experiments. H.E.G., and S.S.G researched data and wrote the manuscript, Q.Y.A-S., Z.N.S., C.G.-W., E.L.E., and M.F.M. researched data. G.H. researched data and reviewed the manuscript. X.T., and L.Y. designed and carried out the experiment with adenovirus injection, and reviewed the manuscript. S.M.N. was responsible for study design, conceptualization, data analysis and results interpretation, and reviewing the manuscript. S.M.N. had full access to all the data of the study and takes responsibility for the integrity and accuracy of data analysis and the decision to submit and publish the manuscript.

Acknowledgements

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Conflict of Interest

No conflict of interest relevant to this work was reported.
Abbreviations: Ad-GFP, adenovirus construct bearing GFP control cDNA; Ad-rWT, adenovirus construct bearing wild-type rat CEACAM1 cDNA; Ad-rSA, adenovirus construct bearing the S503A phosphorylation-defective CEACAM1 mutant cDNA; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; Ceacam1, mRNA transcripts of CEACAM1; Ceacam1, mouse gene encoding CEACAM1 protein; Cc1+/–, mice with global deletion of Ceacam1 gene; CPT-1α, carnitine palmitoyltransferase-1α; Fasn, mouse Fatty acid synthase; FATP, fatty acid transport protein; HF, high-fat diet; NEFA, non-esterified fatty acids; RD, regular standard diet; SREBP-1c, sterol regulatory element binding protein-1c; TNFα, tumor necrosis factor alpha.
Figure Legends

Figure 1: Effect of nicotinic acid on fat accumulation, fibrosis and inflammation in white adipose tissue. (A) H&E staining of the white adipose tissue (WAT) from mice fed ad libitum either with a standard regular diet (RD) or a high-fat diet (HF) for 30 days. In the last 2 days of feeding, RD-fed mice were administered a once daily intraperitoneal injection of saline (S) while HF-fed mice were treated with either saline or Nicotinic Acid (NA) (n>6 mice/feeding/treatment group). (B) Trichrome C (Trich-C) stain was used to assess fibrosis in WAT. Black staining indicates nuclei. Cytoplasm and Fibers are in red. Collagen is shown in blue. Representative images from 3 sections/mouse are shown (20x magnification). (C) Whole WAT was stained with BODIPY 558/568 to detect lipid (gray) for staining of adipocytes and with anti-F4/80 to detect macrophages (green). Nuclei from cells, including macrophages and adipocytes, were stained with DAPI (blue). All images were captured using LSCM with a 63x objective and are 2D projections of a 3D image z-stack. Increased DAPI staining indicating cellular inflammation is observed in HF-NA (iii) compared to RD-S (i) or HF-S (ii) treated animals. F4/80 staining was elevated in HF-NA (ix) compared to HF-S (viii) indicating increased inflammation in HF-NA treated mice, while there was no F4/80 staining in RD-S. The overlay shows that nuclei and macrophages surrounding adipocytes are increased in the HF-NA treated mice (xii vs xi) indicating increased inflammation in the HF-NA treated mice compared to the HF-S treated or RD-S treated mice. Representative images from three sections per mouse per each staining are shown. The scale bar on the last panel of each stain represents scale bar in all other panels per stain.
Figure 2: Effect of nicotinic acid on metabolism and insulin signaling. (A) (i) RD-fed mice were injected with saline (RD-S, white square), and HF-fed mice with saline (HF-S, black square) or nicotinic acid (HF-NA, gray circle) before intra-peritoneal insulin tolerance test was carried out to measure glucose disposal at 0–180 min. (ii) The area under the curve (AUC) was calculated. n= 6-10 mice/feeding/treatment group. Values are expressed as mean ± SEM. *P<0.05 vs RD-S (white bar), †P<0.05 HF-NA (gray bar) vs HF-S (black bar). (B) At the end of feeding/treatment period, mice were fasted overnight (F-white bar) and then refed (RF) for 7h to: (i) measure plasma insulin level. Western analysis in liver (ii) and WAT lysates (iii) was carried out by immunoblotting (Ib) with α-phospho-Akt (α-pAkt) antibodies, followed by reimmunoblotting (re-Ib) with α-Akt antibody to normalize per total Akt loaded. Gels represent 2 independent experiments on 2 mice/feeding/treatment group. (C) (i) Liver lysates were analyzed by Western Blotting, probed with α-CEACAM1 (α-CC1), followed by α-Actin antibody. (ii) Hepatocytes were isolated from wild-type mice and treated with either BSA or with BSA-coupled fatty acid mixture (FA) (0.1 mM), lysed and analyzed by Western blot, using immunoblotting (Ib) with α-CC1, followed by α-GAPDH antibody for normalization. Gel represents 3 independent experiments. (D) Constructs from the mouse promoter bearing the wild-type sequence from nt −1100 to +30 or a block mutation of the only active PPRE/RXR site located between nt −557 and −543 were generated, subcloned into the pGL4.10 promoterless plasmid before their Luciferase activity in response to ethanol (Veh) (white bars) or FA (0.1mM) (black bars) was determined in mouse Hepa1-6 cells. As a negative control, cells were transfected with the empty pGL4.10. The experiment
was performed in quadruplet several times. Luciferase light units were expressed as mean ± SD in relative light units (RLU). The graph represents typical results from several separate experiments. *P<0.05 vs Veh-treated cells (black vs white bar per each construct).

Figure 3: Effect of nicotinic acid on lipid metabolism in liver. (A) Liver histology was assessed by H&E stained sections (n= 5 mice/feeding/treatment group). HF-S mice exhibit micro-vesicular lipid infiltration alternating with normal liver parenchyma. HF-NA manifested a normal histology like that of RD-S. Representative images from 3 sections/mouse are shown (20x magnification). (B) Hepatic fatty acid oxidation was determined in 4 mice/feeding/treatment group. Experiments were repeated at least 3 times. Values are expressed as mean ± SEM. *P<0.05 vs RD-S. (C) (i) Fatty acid synthase (Fasn) enzymatic activity was measured in the liver by [14C]-malonyl-CoA incorporation on mice that had been fasted overnight (F) and refed for 7h (RF). n= 5/feeding/treatment group). Each assay was performed in triplicate. Values are expressed as mean ± SEM. *P<0.05 vs F/each treatment group, †P<0.05 vs F or RF in other treatment groups. (ii) Western analysis of Fasn (normalized to GAPDH) and phosphorylated CEACAM1 normalized to total CEACAM1 in liver lysates. (iii) Co-immunoprecipitation (Co-IP) analysis of Fasn binding to CEACAM1 was carried out by immunoprecipitating (IP) with α-Fasn antibody followed by immunoblotting (Ib) with α-
CC1 antibody. Gels represent more than 2 separate experiments.

Figure 4: Effect of adenoviral-mediated redelivery of CEACAM1 on glucose and insulin intolerance caused by high-fat diet. (A) (i) Western analysis of rat and mouse CEACAM1 expression (rCC1 and mCC1, respectively) in lysates from liver, WAT and sk. muscle of 2 month-old RD-fed wild-type mice 3 days post-injection with Ad-GFP, Ad-rat wild-type CEACAM (Ad-rWT) and Ad-rat serine-to-alanine CEACAM1 mutant (Ad-rSA). Membranes were reprobed with α-GAPDH antibody for normalization. (ii) Western analysis (n= 2/each group) on liver lysates from mice fed with RD or HF for 41 days and injected with adenoviral particles in the last 21 days of feeding. (iii) qRT-PCR analysis of liver lysates to measure rat and mouse Ceacam1 mRNA levels normalized to Gapdh (n= 5/each group in duplicate). (B) (i) Intraperitoneal insulin tolerance and (ii) glucose tolerance tests were carried out 13 and 16 days, respectively, after being injected with Ad-GFP [black (RD) and blue (HF)], Ad-rWT (green) and Ad-rSA (red) (n= 6-8/per group). The area under the curve was measured and presented in graphs on the right. Values are expressed as means ± SEM. *P<0.05 vs RD-fed; †P<0.05 vs HF-GFP.

Figure 5: Effect of adenoviral-mediated redelivery of CEACAM1 on diet-induced lipid alterations. (A) (i) Western analysis of Fasn levels in the liver lysates from mice fed with RD or HF for 41 days and injected with adenoviral particles in the last 21 days of feeding. (ii) Fasn activity was assayed in liver lysates of RD-GFP (back), HF-GFP (blue), HF-rWT (green) or HF-rSA (red) (n= 6-8/per group). Values are expressed as mean ± SEM. *P<0.05 vs RD-GFP; †P<0.05 vs. HF-GFP. (B) Liver histology was
assessed by H&E stained sections (n= 5/each group). Whereas HF-GFP and HF-rSA exhibited micro-vesicular lipid infiltration alternating with normal liver parenchyma, RD-GFP and HF-rWT exhibited normal histology. Representative images from 3 sections/mouse are shown (40x magnification). (C) H&E stain on WAT sections (n= 5/each group). HF diet caused enlarged adipocytes relative to RD-fed mice (HF-GFP vs RD-GFP), as shown by the adipocyte size in the accompanying bar graph. This was reversed by Ad-rWT, but not Ad-rSA, injection. Representative images from 3 sections/mouse are shown (20x magnification), and values of adipocyte size are expressed as mean ± SEM. *P<0.05 vs RD-GFP; †P<0.05 vs. HF-GFP.

**Figure 6: Effect of adenoviral-mediated redelivery of CEACAM1 on diet-induced energy imbalance.** Mice were individually caged (n= 4 per group), fed ad libitum and subjected to indirect calorimetry analysis in a 24 h-period for 5 days to measure: (A) Daily food intake; (B) Heat production (energy expenditure) (kcal/h/kg lean mass). (C) VCO₂ production and VO₂ consumption (mg/h/kg lean mass) to calculate RER as VCO₂/VO₂ ratio, and (D) Spontaneous locomotor activity along the X,Y and Z axes (counts/day). Values are expressed as mean ± SEM of each time interval in the last 3 days. *P<0.05 vs RD-GFP per cycle; †P<0.05 vs. HF-GFP per cycle.
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Park, S. Y., Y. R. Cho, H. J. Kim, E. G. Hong, T. Higashimori, S. J. Lee, I. J. Goldberg,


Table 1: Effect of nicotinic acid on plasma and tissue biochemistry

<table>
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<th>RD-S</th>
<th>HF-S</th>
<th>HF-NA</th>
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<tr>
<td>Body weight (BW, g)</td>
<td>24.7 ± 0.9</td>
<td>29.2 ± 1.0</td>
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<td>% Fat mass (NMR)</td>
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<td>% Lean mass (NMR)</td>
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<td>% Brown adipose tissue (BAT/BW)</td>
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<td>8.5 ± 1.1</td>
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<td>Hepatic triacylglycerol (μg/mg)</td>
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<td>Plasma insulin (Ins) (pM)</td>
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<td>Plasma C-peptide (pM)</td>
<td>475 ± 98.</td>
<td>514 ± 123.</td>
<td>548 ± 114.</td>
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<td>Plasma C-peptide/Ins Molar Ratio</td>
<td>6.0 ± 0.4</td>
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<td>Fasting blood glucose (mg/dl)</td>
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<td>99. ± 12.</td>
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<td>Fed blood glucose (mg/dl)</td>
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<td>127. ± 3.</td>
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<tr>
<td>Fed plasma leptin (ng/ml)</td>
<td>0.7 ± 0.1</td>
<td>3.5 ± 1.1</td>
<td>2.8 ± 0.4</td>
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Male mice (n=6-10/feeding/treatment group) were fed RD or HF diet for 30 days. In the last 2 days of feeding, mice were treated with nicotinic acid (NA), and their body fat composition evaluated by nuclear magnetic resonance (NMR; Bruker Optics). Except for leptin and fed blood glucose levels, mice were fasted overnight for 18h and their tissues extracted to assess visceral and brown fat mass relative to body weight (BW), and blood was drawn to determine plasma levels of non-esterified fatty acids (NEFA), triacylglycerol, insulin (Ins) and C-peptide levels. Values are expressed as mean ± SEM.

\( ^a P < 0.05 \) vs RD-S; \(^b P < 0.05 \) HF-NA vs HF-S.
Table 2: Effect of adenoviral-mediated CEACAM1 redelivery to the liver on plasma and tissue biochemistry

<table>
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<th>HF-rWT</th>
<th>HF-rSA</th>
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<td>Body weight (g)</td>
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<td>% Visceral fat (WAT/BW)</td>
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<td>2.6±0.4</td>
<td>2.1±0.1</td>
<td>3.4±0.3</td>
</tr>
<tr>
<td>% BAT/BW</td>
<td>0.31±0.02</td>
<td>0.25±0.02</td>
<td>0.30±0.03</td>
<td>0.23±0.01</td>
</tr>
<tr>
<td>Hepatic triacylglycerol (μg/mg)</td>
<td>55.4±8.2</td>
<td>104.2±18.1</td>
<td>54.3±6.9</td>
<td>98.6±15.6</td>
</tr>
<tr>
<td>Plasma NEFA (mEq/l)</td>
<td>0.4±0.0</td>
<td>0.5±0.0</td>
<td>0.4±0.0</td>
<td>0.5±0.0</td>
</tr>
<tr>
<td>Plasma triacylglycerol (mg/dl)</td>
<td>48.5±3.8</td>
<td>44.7±3.5</td>
<td>51.4±4.0</td>
<td>49.6±3.0</td>
</tr>
<tr>
<td>Plasma insulin (pM)</td>
<td>28.8±2.4</td>
<td>52.3±8.5</td>
<td>32.1±3.5</td>
<td>57.0±8.6</td>
</tr>
<tr>
<td>Plasma C-peptide (pM)</td>
<td>193.6±8.6</td>
<td>307.8±58.3</td>
<td>199.1±7.7</td>
<td>297.4±32.5</td>
</tr>
<tr>
<td>Steady-state C-peptide/Insulin</td>
<td>8.6±0.6</td>
<td>5.2±0.3</td>
<td>7.0±0.8</td>
<td>4.4±0.3</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mg/dl)</td>
<td>70.±5</td>
<td>71.±6.</td>
<td>73.±4</td>
<td>83.±7</td>
</tr>
<tr>
<td>Fed Blood Glucose (mg/dl)</td>
<td>112.4±3.7</td>
<td>128.3±4.4</td>
<td>117.3±2.0</td>
<td>131.1±4.7</td>
</tr>
<tr>
<td>Plasma FGF21 (pmol/l)</td>
<td>11.2±2.2</td>
<td>5.3±1.1</td>
<td>16.2±3.0</td>
<td>6.0±1.2</td>
</tr>
</tbody>
</table>

Male mice (n=6-10/feeding/treatment group) were fed RD or HF diet for 20 days before being injected through the tail vein with comparable amounts of adenoviral particles of Ad-GFP (as control); Ad-rat wild-type CEACAM1 (Ad-rWT) and Ad-rat S503A phosphorylation-defective CEACAM1 mutant (Ad-rSA) while they were maintained on RD or HF diet. At the end of the experiments (21 days post-injection), mice were sacrificed and tissues and blood removed to carry out the same analyses described in the legend to Table 1. Values are expressed as mean ± SEM. \(^aP<0.05\) vs RD-GFP; \(^bP<0.05\) vs HF-GFP and \(^cP<0.05\) HF-rSA vs HF-rWT.
Figure 1

A. H&E

B. Trich-C

C. Immunofluorescence

- RD-S
- HF-S
- HF-NA
Figure 2

A. Insulin tolerance test

- Glucose (% Basal)
  - Graph showing glucose levels over time (Post-Insulin ip (Min))

- AUC (x10^-2)
  - Bar graph showing AUC values for different conditions

B. Insulin signaling

- Insulin (x10^-2 pM)
  - Graph showing insulin levels

- Liver
  - Western blots for α-CC1 and α-Actin

- WAT
  - Western blots for α-pAkt and α-Akt

C. CEACAM1 levels

- Liver
  - Western blots for α-CC1 and α-Actin

- Primary Hepatocytes
  - Western blots for α-CC1 and α-GAPDH

D. Ceacam1 promoter Activity

- Schematic showing promoter activity

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Figure 3

**A. H&E staining**

**B. Fatty acid oxidation**

**C. Regulation of Fasn activity**

1. Fasn Activity

2. l:α-Fasn

3. relb:α-GAPDH

4. l:α-pCC1

5. relb:α-CC1

6. Co-IP

IP:α-Fasn

lb:α-CC1

relb:α-Fasn
A. CEACAM1 adenoviral-redelivery to the Liver

\[ \text{i. WB: day 3} \]

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>WAT</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GFP</strong></td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>WT</strong></td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>rSA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- lb: α-rCC1
- relb: α-mCC1
- relb: α-GAPDH

\[ \text{ii. WB: day 21} \]

<table>
<thead>
<tr>
<th></th>
<th>RD-GFP</th>
<th>HF-GFP</th>
<th>HF-rWT</th>
<th>HF-rSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

- lb: α-rCC1
- relb: α-mCC1
- relb: α-GAPDH

\[ \text{iii. qRT-PCR: day 21} \]

- rCc1/Gapdh
- mCc1/Gapdh

B. Insulin and glucose tolerance tests

\[ \text{i. Glucose (% Basal)} \]

- HF-rSA
- HF-GFP
- RD-GFP
- HF-rWT

\[ \text{AUC (x10-3)} \]

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

\[ \text{AUC (x10-3)} \]
Figure 5

A. Fasn levels and activity in liver

i. WB

<table>
<thead>
<tr>
<th></th>
<th>RD-GFP</th>
<th>HF-GFP</th>
<th>HF-rWT</th>
<th>HF-rSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>lb:α-Fasn</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>relb:α-GAPDH</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

ii. Fasn Activity

(\(^1^4\)C) Malonyl-CoA Incorporation

<table>
<thead>
<tr>
<th></th>
<th>RD-GFP</th>
<th>HF-GFP</th>
<th>HF-rWT</th>
<th>HF-rSA</th>
</tr>
</thead>
</table>

B. H&E stain in liver

C. H&E stain in WAT

![Images of H&E stain for liver and WAT]
Figure 6

A. Daily food intake

![Figure A: Daily food intake graph with comparison between light and dark conditions.](image)

B. Heat production

![Figure B: Heat production graph showing kcal/h/kg.](image)

C. RER (VCO2/VO2)

![Figure C: RER (VCO2/VO2) graph.](image)

D. Spontaneous locomotor activity

![Figure D: Spontaneous locomotor activity graph with various conditions.](image)
Chapter 6

Fenofibrate Decreases Insulin Clearance and Insulin Secretion to Maintain Insulin Sensitivity

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¹ Authors contributed equally to these studies
Abstract

High fat diet reduces the expression of CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1), a transmembrane glycoprotein that promotes insulin clearance and downregulates fatty acid synthase activity in the liver upon its phosphorylation by the insulin receptor. Because peroxisome proliferator-activated receptor α (PPARα) transcriptionally suppresses CEACAM1 expression, we herein examined whether high fat downregulates CEACAM1 expression in a PPARα-dependent mechanism. By activating PPARα, the lipid-lowering drug fenofibrate reverses dyslipidemia and improves insulin sensitivity in type 2 diabetes in part by promoting fatty acid oxidation. Despite reducing glucose-stimulated insulin secretion, fenofibrate treatment does not result in insulin insufficiency. To examine whether this is mediated by a parallel decrease in CEACAM1-dependent hepatic insulin clearance pathways, we fed wild-type and Pparα−/− null mice a high fat diet supplemented with either fenofibrate or Wy14643, a selective PPARα agonist, and examined their effect on insulin metabolism and action. We demonstrated that the decrease in insulin secretion by fenofibrate and Wy14643 is accompanied by reduction in insulin clearance in wild-type but not Pparα−/− mice, thereby maintaining normoinsulinemia and insulin sensitivity despite continuous high fat intake. Intact insulin secretion in L-CC1 mice with protected hepatic insulin clearance and CEACAM1 levels provides in vivo evidence that insulin secretion responds to changes in insulin clearance to maintain physiologic insulin and glucose homeostasis. These results also emphasize the relevant role of hepatic insulin extraction in regulating insulin sensitivity.
Introduction

Insulin regulates glucose homeostasis by inhibiting hepatic gluconeogenesis and promoting glucose disposal. Numerous environmental cues, including dietary, hormonal, and stress factors play a key role in regulating insulin response. Peripheral insulin resistance, manifested by dysregulated glucose and lipid metabolism, leads to compensatory increase in insulin secretion from pancreatic β-cells. Persistence of insulin resistance eventually causes β-cell failure and subsequently, overt type 2 diabetes.

Type 2 diabetes is a metabolic disease characterized by insulin resistance, dyslipidemia, and cardiovascular complications. Abdominal obesity, ectopic triacylglycerol accumulation, and atherogenic dyslipidemia are common clinical manifestations of diabetes. Fibrates are lipid-lowering drugs that are commonly used to treat hypertriglyceridemia, primary hypercholesterolemia, and mixed dyslipidemia in cardiometabolic diseases (Kliewer et al., 2001, Shipman et al., 2016). Fenofibrate treatment decreases lipid content in the liver and skeletal muscle and decreases apolipoprotein B and very LDL synthesis to improve very LDL catabolism in addition to increasing HDL level (Van der Hoogt et al., 2007). Like other members of this class of drugs, fenofibrate activates PPARα to promote fatty acid β-oxidation. It also regulates oxidation indirectly by activating AMP-activated protein kinase that reduces the level of malonyl-CoA, a precursor of palmitate synthesis via fatty acid synthase, and an inhibitor of carnitine palmitoyltransferase 1 that catalyzes fatty acid translocation to the mitochondria to undergo oxidation (Sozio et al., 2010).

We have recently shown that PPARα activation at fasting suppresses the transcription of CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule) (Ramakrishnan et
al., 2016). CEACAM1 is a membrane glycoprotein that promotes hepatic insulin endocytosis and targeting to the degradation process upon its phosphorylation by the insulin receptor (Formisano et al., 1995) to promote its clearance and maintain systemic insulin sensitivity (Poy et al., 2002, DeAngelis et al., 2008). Additionally, CEACAM1 down-regulates hepatic fatty acid synthase activity in response to an acute rise in insulin (Najjar et al., 2005), thus contributing to the regulation of the relative level of malonyl-CoA to long chain fatty acyl-CoA, an endogenous ligand of PPARα, and subsequently, to fatty acid oxidation during the fasting-refeeding transition (Ramakrishnan et al., 2016).

Using mice with null deletion of Ppara (Ppara−/−), several laboratories have shown that PPARα activation by fatty acids plays an important role in insulin resistance, hepatic steatosis, and dyslipidemia in response to high fat diet (Patsouris et al., 2006, Leone et al., 1999). High fat diet reduces hepatic Ceacam1 mRNA levels, whereas its forced transgenic expression in liver limits diet-induced resistance and hepatic steatosis (Al-Share et al., 2015). This supports an important role for defective CEACAM1-dependent insulin clearance mechanisms in the metabolic dysregulations caused by a high fat diet. As in fasting, increased dietary fat supply drives redistribution of fatty acids released from white adipose tissue during lipolysis to the liver to undergo oxidation. Thus, we investigated whether high fat diet down-regulates hepatic CEACAM1 expression in a PPARα-dependent manner. Because fenofibrate activates PPARα-mediated fatty acid oxidation, we also investigated whether it too modulates hepatic CEACAM1 expression and whether this mediates its therapeutic effect on insulin metabolism and action and limits hepatic steatosis under high fat feeding conditions.
Experimental Procedures

Mice Feeding—WT and Pparaα−/− null mice were propagated on the C57BL/6 background (Taconic Biosciences, Cambridge City, IN). The mice were kept in a 12-h dark/light cycle. As previously described (Al-Share et al., 2015), male mice (3 months of age) were fed ad libitum either RD deriving 12:66:22% calories from fat:carbohydrate:protein or a HF deriving 45:35:20% calories from fat:carbohydrate:protein (Research Diets), respectively. The dietary fat composition of HF is 36.3% SFA, 45.3% MUFA, and 18.5% Ω6PUFA. HF contains mostly sucrose with very little fibers, as opposed to RD, which is high in fibers with insignificant amount of sucrose.

Unless otherwise mentioned, the mice were fed HF for 9 weeks. Thereafter, the mice were fed a chow diet powdered and mixed in a geometric proportion with 0.1% (w/w) of Wy14643 (Enzo Life Sciences, Farmingdale, NY) for 10 days or with 0.1% fenofibrate (Sigma) for 21 days. The institutional animal care and utilization committee approved all of the procedures.

Body Composition—Whole body composition was evaluated by NMR (Bruker Optics).

Metabolic Parameters—At the end of the feeding period, the mice were fasted before blood was drawn, and tissues were extracted to measure metabolic parameters. Whole blood glucose measurements were made with a glucometer (Accu-check; Roche). Retro-orbital venous blood was drawn at 1100 h from overnight fasted mice to assess plasma insulin and C-peptide levels by radioimmunoassay (Linco Research Inc., St. Charles, MO), FFA (NEFA C; Wako), and triacylglycerol (Pointe Scientific Triglyceride, Canton, MI). Hepatic triacylglycerol was measured as described (Al-Share et al., 2015). Insulin clearance was measured as the steady state C-peptide/insulin molar ratio. This approach
has been used to assess insulin clearance, being consistent with measuring the rate of disappearance of intravenously injected $[^{125}]I$ insulin from the circulation (Poy et al., 2002).

**Glucose and Insulin Tolerance Tests**—Insulin and glucose tolerance tests were conducted 3 and 7 days after initiation of Wy14643 supplementation, respectively. For fenofibrate, they were conducted 9 and 13 days after initiation of treatment. On the day of the experiment, awake mice were fasted for 6 h starting at 0800 h, injected intraperitoneally with either 1.5 g/kg of body weight (50%) dextrose solution (glucose tolerance) or regular human insulin (Novo Nordisk; 0.75 unit/kg of body weight) (insulin tolerance) and subjected to blood drawing from tail vein to measure glucose at 0–180 min.

**Glucose-stimulated Insulin Secretion**—A week after the insulin tolerance test was performed, mice were fasted overnight, anesthetized using pentobarbital at 55 mg/kg of body weight, and injected with glucose at 3 g/kg of body weight, and blood was drawn from the retro-orbital sinus to assess insulin levels at 0–30 min after glucose injection.

**Primary Hepatocytes and Treatment with Fatty Acids**—The liver of anesthetized mice was perfused (1 ml/min) with collagenase type II solution (1 mg/ml) (Worthington), as described (Al-Share et al., 2015). Hepatocytes were dispensed in Williams E complete medium containing 10 mM lactate, 10 nM dexamethasone, 100 nM insulin, 10% FBS, and 1% penicillin-streptomycin before being plated onto 6-well cell-culture plates at $2.5 \times 10^5$/well density and incubated at 37 °C for 24 h. The medium was then replaced with phenol red-free Williams E medium (Gibco A-12176 – 01) supplemented with 10% dialyzed FBS and 1% penicillin-streptomycin for 24 h. The cells were then incubated for 24 h with 0.1 mM of individual fatty acids PA (SFA; C16:0), OA (MUFA; n-9, C18:1),
and LA (Ω6 PUFA; n-6, C18:2)) or with 0.1 mM of fatty acid mixture (0.035 mM PA, 0.045 mM OA, and 0.02 mM LA) that were reconstituted in EtOH and coupled to 2 mM insulin-free BSA at a ratio of 1:5.

Immunoﬂuorescence Analysis—The pancreas was isolated and fixed immediately in PBS-buffered formalin and embedded in paraffin. Hematoxylin and eosin staining was performed in formalin-fixed paraffin sections. For immunostaining, paraffin-embedded tissues sections were deparaffinized in xylene, rehydrated in gradient ethanol, blocked with 10% goat serum for 1 h, and then probed overnight at 4 °C with anti-insulin antibody (Abcam). Following three washes, Alexa Fluor-labeled secondary antibody was added to the tissue sections, and images were taken using a fluorescent microscope.

Isolation and Glucose-stimulating Insulin Secretion in Pancreatic Islets—Pancreatic islets were isolated as previously described (Liu et al., 2007). Briefly, the animals were anesthetized using pentobarbital at 55 mg/kg of body weight, and pancreata were digested with collagenase P (2 mg/ml) for 30 min at 37 °C. Islets were pelleted by centrifugation at 1500 rpm for 5 min. The pellets were washed twice with ice-cold PBS and then resuspended in ice-cold PBS before being picked using microscope. Islets were then incubated overnight in RPMI 1640 containing 11.1 mM glucose and 10% FBS to recover. Islets were again handpicked, and 15 islets/group were incubated for 2 h at 37 °C in KRBH buffer (129 mM NaCl, 5 mM NaHCO3, 4.8 mM KCl, 1.2 mM KH2PO4, 2.5 mM CaCl2, 1.2 mM MgSO4, and 10 mM Hepes, pH 7.4, containing 2.8 mM glucose plus 0.2% radioimmunoassay grade BSA). Glucose-stimulated insulin secretion (GSIS) was performed by incubating islets in KRBH containing 16.7 mM glucose for 1 h. The media were collected, and the insulin release in the media was measured using sensitive rat
insulin kit (Millipore Inc.). Islets were lysed using lysis buffer, and the GSIS was normalized to the protein content in islets.

**Western Analysis**—Protein from lysates were analyzed by 7% SDS-PAGE. The membranes were cut in half to immunoblot the upper half with custom-made polyclonal antibodies against mouse CEACAM1 (Al-Share et al., 2015) and the lower half with monoclonal antibodies against actin, tubulin, or GAPDH (Sigma-Aldrich or Santa Cruz) to normalize for protein loading. In some experiments, the lower half was reprobed with a polyclonal antibody against CD36 (Santa Cruz). The blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences) antibodies prior to detection by enhanced chemiluminescence (Amersham Biosciences). Odyssey was used to detect proteins in some gels. Band intensity was quantified using ImageJ software.

**Semi-quantitative Real Time RT-PCR**—Total RNA was extracted with a PerfectPure RNA tissue kit (5 Prime) per manufacturer’s protocol. cDNA was synthesized using 1 µg of total RNA with iScript™ cDNA synthesis kit (Bio-Rad), using 1 µg of total RNA and oligo (dT) primers. cDNA was evaluated with quantitative RT-PCR (Step One Plus, Applied Biosystems). The primer sequences for Ceacam1 were 5'-AATCTGCCCCTGG-CGCTTGGAGCC-3' (forward) and 5'-AAATCGCACAGTC-GCCTGAGTACG-3' (reverse), and those for 18S were 5'-TTCGAACGTCTGCCCTATCAA-3' (forward) and 5'-ATG-GTGGCACGCGACTA-3' (reverse). The relative amount of mRNA was normalized to 18S. The results are expressed as fold change in gene expression.

**Statistical Analysis**—The data were analyzed with SPSS software by two-way analysis of variance with Bonferroni correction. $p < 0.05$ were statistically significant.
Results

High Fat Diet Does Not Modulate Hepatic CEACAM1 Expression in Ppara<sup>−/−</sup> Mice—High fat diet (HF) reduces hepatic CEACAM1 mRNA and protein levels (Al-Share et al., 2015). To investigate dependence on PPARα activation, we examined whether HF adversely affects CEACAM1 expression in Ppara<sup>−/−</sup> mice. Immunoblotting with α-CEACAM1 antibody (α-CC1) revealed reduction in CEACAM1 protein content in liver lysates of HF-fed WT mice relative to mice fed a regular chow diet (RD) (Fig. 1A), as expected from previous reports (Al-Share et al., 2015). In contrast, HF failed to modulate CEACAM1 protein levels in Ppara<sup>−/−</sup> mice (Fig. 1A, HF versus RD).

We then tested the effect of a representative fatty acid from each of the classes in HF on CEACAM1 expression in primary hepatocytes. These include palmitic acid (SFA), oleic (MUFA), and linoleic acid (Ω6 PUFA), individually (0.1 mM each) or in combination at 35:45:20, respectively. As Fig. 1B (panel i) shows, treatment with oleic acid (OA), linoleic acid (LA), and the fatty acid mixture (Mix) significantly decreased Ceacam1 mRNA levels by comparison with BSA treatment (−) in primary hepatocytes isolated from WT but not Ppara<sup>−/−</sup> mice. In contrast, palmatic acid (PA), a weaker agonist of PPARα than oleic and linoleic acids (Kliwer et al., 1997, Forman et al., 1997, Hostetler et al., 2006) did not reduce Ceacam1 mRNA level in primary hepatocytes derived from either genotype (Fig. 1B, panel i). Instead, it markedly induced it in cells derived from Ppara<sup>−/−</sup> mice (Fig. 1B, panel i). The fatty acid mixture also increased Ceacam1 mRNA levels relative to BSA, albeit to a lower extent than palmatic acid, in Ppara<sup>−/−</sup> mice (Fig. 1B, panel i).

At the protein level, oleic and linoleic acids reduced CEACAM1 markedly by 12 h of
treatment in cells isolated from WT mice (Fig. 1B, panel ii, parts a and b), but not from \( \text{Ppara}^{-/-} \) mutants even after 24 h of treatment (Fig. 1B, panel ii, part b). In contrast, palmitic acid did not modify CEACAM1 expression in either genotype. Treating with the fatty acid mixture lowered CEACAM1 protein levels in WT, but not \( \text{Ppara}^{-/-} \) hepatocytes (Fig. 1B, panel ii, part b).

**Differential Metabolic Effect of High Fat Diet in WT and Pparα-/- Mice**—In agreement with other reports (Kersten et al., 1999), \( \text{Pparα}^{-/-} \) mice displayed hypoglycemia compared with WT mice during a 48-h fasting period, irrespective of the diet (not shown). Relative to RD, HF intake caused glucose and insulin intolerance in WT mice (Fig. 2, A and B, respectively). On RD diet, \( \text{Pparα}^{-/-} \) mice were more glucose-tolerant and equally insulin-tolerant relative to WT mice (Fig. 2, A and B, respectively), and HF did not significantly modify their glucose and insulin tolerance, as it did to their WT counterparts. Moreover, RD-fed \( \text{Pparα}^{-/-} \) mice displayed random normoglycemia compared with RD-fed WT mice (130.0 ± 4.0 RD-fed \( \text{Pparα}^{-/-} \) versus 125.0 ± 3.0 mg/dl in RD-fed WT). In contrast to WT that exhibited random hyperglycemia when fed a HF diet (150.0 ± 2.0 in HF-fed WT versus 125.0 ± 3.0 in RD-fed WT; \( p <0.05 \)), HF did not elevate random glucose levels in \( \text{Pparα}^{-/-} \) mice (131.0 ± 3.0 in HF-fed versus 130.0 ± 4.0 in RD-fed). This demonstrates that \( \text{Pparα}^{-/-} \) deletion confers protection against diet-induced insulin resistance, as has been previously shown (Guerre-Millo et al., 2001, Bernal-Mizrachi et al., 2003).

As expected from the negative effect of PPARα activation on insulin secretion (Gremlich et al., 2005, Rubi et al., 2002), glucose induced a more robust acute rise in insulin release in RD-fed null mice relative to their RD-fed WT counterparts (Fig. 2C). Whereas HF did not further stimulate insulin release in response to glucose in the null mouse, it induced
more acute release of insulin in wild types (Fig. 2C), likely to compensate for insulin resistance in these mice.

Consistent with the lack of HF effect on hepatic CEACAM1 levels in \( Ppara^{-/-} \), HF intake did not reduce insulin clearance in these mice, as it did to wild types (C-peptide/insulin molar ratio; Tables 1 and 2). This was associated with normal plasma insulin levels (Tables 1 and 2) and glucose tolerance (Fig. 2A) in HF-fed mice relative to RD-fed \( Ppara^{-/-} \) mice. Of note, RD-fed \( Ppara^{-/-} \) mice maintained normal insulin sensitivity despite a marked elevation in hepatic triacylglycerol level (by 2-fold) (Tables 1 and 2), likely emerging from compromised fatty acid -oxidation, per other reports (Sozio et al., 2010, Montagner et al., 2016), and by reduced output, as shown by the 1.5–2-fold lower plasma triacylglycerol level relative to RD-fed WT mice (Tables 1 and 2). Increased hepatic steatosis could contribute to the higher body weight and total fat mass in \( Ppara^{-/-} \) mice (Table 1), as expected (Kersten et al., 1999). Together, the data propose that protected hepatic CEACAM1 levels and function could contribute strongly to sustained insulin sensitivity in \( Ppara^{-/-} \) mice despite continuous HF intake.

**Effect of Wy14643 on Diet-induced Changes in Insulin Metabolism and Action**— Because the PPAR\( \alpha \) selective agonist Wy14643 suppresses Ceacam1 transcription to maintain physiologic insulin metabolism in the face of reduced insulin secretion (Ramakrishnan et al., 2016), we then investigated whether it can reverse the negative effect of HF on insulin metabolism and action. As shown in Fig. 3A, supplementing HF with 0.1% Wy14643 restored glucose tolerance and insulin sensitivity in HF-fed WT mice (Fig. 3, A and B, respectively). This was accompanied by a reduction in hepatic steatosis and normalization of plasma triacylglycerol and NEFA levels, in parallel to the reversal of
visceral obesity and body weight gain (Table 1). In contrast to WT mice, Wy14643 supplementation did not affect hepatic triacylglycerol level (Table 1), nor did it modulate significantly glucose and insulin tolerance in Ppara<sup>−/−</sup> mice (Fig. 3, A and B, respectively). Consistent with the suppressive effect of HF on hepatic CEACAM1 expression (Fig. 1), HF significantly lowered insulin clearance in WT (as assessed by the decrease in the steady state C-peptide/insulin molar ratio) but not Ppara<sup>−/−</sup> mice (Table 1). This contributes to hyperinsulinemia in HF-fed relative to RD-fed WT mice that also manifested higher plasma C-peptide levels, a marker of insulin secretion (Table 1). As expected, Wy14643 supplementation markedly limited the induction of C-peptide levels by HF feeding in these WT mice (by 2-fold). Wy14643 supplementation did not significantly improve the steady state C-peptide/insulin molar ratio in HF-fed mice (Table 1: 11.4 ± 0.8 versus 9.1 ± 1.5 in HF-fed WT mice). Sustained low insulin clearance appears to be mediated by the remarkable decrease in CEACAM1 protein level in the liver of mice fed HF supplemented with Wy14643 relative to HF-fed mice, likely resulting from the synergistic down-regulatory effect of HF and Wy14643 on Ceacam1 expression (Fig. 3C). Of note, the detectable basal insulin clearance in these mice is conceivably mediated by insulin receptor phosphorylation, a committed initial step in insulin internalization and degradation under conditions of insulin sensitivity (Carpentier et al., 1992). Together, the data propose that sustained reduction in CEACAM1 supports hepatic fatty acid oxidation and prevents insulin insufficiency in response to Wy14643 supplementation of the high fat diet.

Coordinated Regulation of Insulin and Lipid Metabolism by Fenofibrate—As expected, fenofibrate treatment for 3 weeks reduced hepatic and plasma triacylglycerol content in
WT mice by ~2–4-fold relative to RD-fed mice. This caused reduction in body weight (23.8 ± 0.5 versus 25.6 ± 0.3 g; p <0.05), visceral obesity (1.6 ± 0.5 versus 2.9 ± 0.1%; p <0.05), and, subsequently, plasma NEFA (0.3 ± 0.0 versus 0.7 ± 0.1 mEq/liter; p <0.05) in WT mice. In contrast, fenofibrate failed to modulate these parameters in Ppara<sup>−/−</sup> mice (not shown).

As expected, fenofibrate treatment did not affect glucose tolerance in RD-fed WT or Ppara<sup>−/−</sup> mice (Fig. 4A). In WT, it improved glucose clearance in response to exogenous insulin (Fig. 4B). In fact, in some mice, this caused severe hypoglycemia, prompting us to stop blood drawing 60 min after insulin injection. In Ppara<sup>−/−</sup> mice, however, fenofibrate did not significantly affect insulin tolerance (Fig. 4B).

Similar to our previous observations of a marked reduction in acute phase glucose-stimulated insulin secretion in Wy14643-treated mice (Ramakrishnan et al., 2016), fenofibrate treatment almost completely abolished acute phase insulin secretion in WT, but not Ppara<sup>−/−</sup> mice (Fig. 4C), in parallel to reducing steady state plasma C-peptide levels (145.1 ± 6.0 versus 309.3 ± 32.0 pM in RD-fed WT mice; p <0.05). These data are in agreement with the reported suppressive effect of fenofibrate on insulin secretion in monosodium glutamate-induced obese rats (Liu et al., 2011).

Changes in insulin secretion are often associated with changes in islet size and mass (Kulkarni 2004). Fenofibrate treatment did not significantly affect the islet size or area in either genotype (Fig. 5A). Immunofluorescence staining of pancreas with polyclonal antibody against insulin revealed a comparable amount of insulin content in the islets of both mouse groups (Fig. 5B).

We then examined whether fenofibrate affects β-cell function by assessing glucose-
stimulated insulin secretion in isolated islets from mice treated with 0.1% fenofibrate for 3 weeks. Normalized to protein content, basal and glucose-stimulated insulin secretion were comparable in total islets isolated from both mouse groups (Fig. 5C). This suggests that the detected negative effect of fenofibrate on insulin secretion was mediated by a cell non-autonomous mechanism.

Despite reduced insulin secretion, fenofibrate-treated WT mice did not develop marked insulin insufficiency, as revealed by normal or slightly reduced fasting insulin levels in some cohorts of mice (58.6 ± 3.2 versus 56.3 ± 4.4 pM in RD-fed WT mice). This protection is likely due to a parallel decrease in insulin clearance (steady state C-peptide/insulin molar ratio) in WT mice (2.8 ± 0.0 versus 4.2 ± 0.3; p 0.05). Similar to Wy14643 (Ramakrishnan et al., 2016), fenofibrate treatment did not modulate insulin and C-peptide levels in Ppara−/− mice. Consistently, Western blotting analysis of liver lysates revealed a 50% reduction in CEACAM1 protein levels in WT, but not Ppara−/− mice, in response to Ppara activation, as assessed by elevation in the protein content of its transcriptional target, CD36 (Sato et al., 2002) (Fig. 4D).

To further address the relationship between fenofibrate’s regulation of insulin clearance and secretion, we then examined its effect on insulin metabolism in L-CC1 mice with liver-specific overexpression of rat CEACAM1 driven by human apolipoprotein A1 promoter (Poy et al., 2002). As Fig. 6 shows, fenofibrate markedly reduced acute phase insulin release in response to glucose (Fig. 6A and accompanying graph depicting the area under the curve), and lowered plasma C-peptide level (Fig. 6B, panel i) in WT, but not L-CC1 mice. Fenofibrate also reduced insulin clearance in WT, but not L-CC1 (Fig. 6B, panel iii), maintaining normal plasma insulin (Fig. 6B, panel ii) and blood glucose
levels in WT animals (Fig. 6C). As expected, fenofibrate reduced hepatic mouse CEACAM1 level in both mouse strains (Fig. 6D).

In contrast, its effect on transgenic rat CEACAM1 was negligible (Fig. 6D), consistent with its positive PPARα-mediated role on human apolipoprotein A1 promoter transcriptional activity (Vu-Dac et al., 1998). Normoinsulinemia and normoglycemia in L-CC1 mice preclude a primary effect of fenofibrate on insulin secretion, because this would be expected to cause insulin insufficiency and hyperglycemia in the face of protected insulin clearance.

Role of Insulin Metabolism in Mediating the Regulation of Metabolic Response to High Fat Diet by Fenofibrate—To assess the effect of fenofibrate on diet-induced obesity, HF was supplemented with 0.1% (w/w) fenofibrate for up to 3 weeks before sacrifice. In WT mice, fenofibrate supplementation significantly lowered hepatic steatosis, in addition to plasma triacylglycerol and NEFA to a level even lower than RD-fed mice (Table 2). It also reversed the HF-induced gain in body weight and in fat mass in WT but not Ppara⁻/⁻ mice (Table 2). Moreover, it restored glucose tolerance (Fig. 7A) and prevented insulin intolerance caused by HF in WT mice (Fig. 7B).

As expected from restored insulin sensitivity, fenofibrate reduced acute phase insulin release in response to glucose in HF-fed mice, as well as RD-fed WT mice (Fig. 7C). It also normalized plasma insulin and C-peptide levels in these mice when fed a HF diet (Table 2).

As with Wy14643 supplementation, decreased insulin secretion was accompanied by a reduction in insulin clearance (assessed by lower steady state C-peptide/insulin molar ratio in mice fed a fenofibrate-supplemented HF diet by comparison with RD-fed mice)
This prevented hypoinsulinemia and hyperglycemia. Reduced insulin clearance was likely mediated by low hepatic CEACAM1 levels in mice fed a fenofibrate-supplemented HF diet relative to HF- and RD-fed mice (Fig. 7D) in response to the combined down-regulatory effect of Ppara agonist and HF on CEACAM1 expression (Ramakrishnan et al., 2016, Al-Share et al., 2015). In contrast to WT, fenofibrate supplementation failed to exert a significant effect on HF-induced metabolic changes in Ppara<sup>−/−</sup> mice (Table 2).

**Discussion**

Fibrates are lipid-lowering drugs that promote triglyceride catabolism, largely by activating PPAR<sub>α</sub>-dependent fatty acid oxidation. In accordance with improved insulin sensitivity, fenofibrate reduces insulin secretion (Liu et al., 2011). We herein examined whether it causes a parallel decrease in insulin clearance and whether this could implicate changes in the hepatic content of CEACAM1, a main chaperone of insulin endocytosis and targeting toward degradation (Najjar 2002). We show that, like Wy14643 (Ramakrishnan et al., 2016), supplementing fat-enriched diets with fenofibrate reduce insulin clearance in parallel to hepatic CEACAM1 expression. Although high fat diet alone causes insulin resistance and hepatic steatosis by a hyperinsulinemia-driven mechanism and thus elicits a compensatory increase in insulin secretion (Al-Share et al., 2015), the PPAR<sub>α</sub> agonists Wy14643 and fenofibrate reverse diet-induced insulin resistance and insulin secretion in parallel to promoting fatty acid oxidation. Their effect appears to be maintained, at least in part, by low hepatic CEACAM1 levels and subsequently reduced insulin clearance to sustain physiologic insulin metabolism, while
maintaining fatty acid oxidation, because of alleviating the negative effect of CEACAM1 on fatty acid synthase activity and subsequently reducing inhibition of fatty acid oxidation by malonyl-CoA.

We have shown that high fat intake causes a decline in hepatic CEACAM1 expression at the transcriptional level to cause insulin resistance (Al-Share et al., 2015). The current studies show that this negative effect of dietary fat on CEACAM1 expression is mediated by Ppara activation insofar as 1) high fat diet failed to reduce hepatic CEACAM1 in Ppara$^{-/-}$ mice and 2) fatty acids that are known endogenous ligands of PPARα (MUFA and PUFA) reduce Ceacam1 mRNA and CEACAM1 protein levels in primary hepatocytes derived from wild-type but not Ppara$^{-/-}$ mice.

In a small cohort of seven overweight or obese, non-diabetic humans, oral ingestion of an emulsion containing predominantly either MUFA, PUFA, or SFA at regular intervals for 24 h caused reduction in insulin clearance, with the greatest down-regulatory effect being exerted by PUFA (linoleate) and SFA being the only fat causing insulin resistance (Xiao et al., 2006). Failure of SFA to regulate CEACAM1 expression in isolated primary hepatocytes in the current studies suggests that the reported reduction in insulin clearance in human subjects receiving an SFA emulsion is likely to be a consequence of insulin resistance and associated defect in insulin receptor autophosphorylation, the first committed step in insulin clearance. Moreover, portal delivery of oleate (Yoshii et al., 2006) and intralipid-heparin infusion, which elevated fasting plasma levels of linoleate (PUFA), oleate (MUFA), and palmitate (SFA) by 4-, 2-, and 1.7-fold, respectively, impaired hepatic insulin clearance in dogs (Wiesenthal et al., 1999). More recently, we
observed that a similar intralipid-heparin infusion caused a decline in hepatic insulin clearance in rats, in parallel to a marked decrease in hepatic CEACAM1 protein content (Pereira et al., 2014). Although these studies did not address whether fatty acid activation of PPARα is implicated in the reduction of CEACAM1 expression by intralipid infusion, they further demonstrated the relevant role of hepatic insulin clearance in the metabolic response to elevation in plasma free fatty acids (Ader et al., 2014).

The current studies show that prolonged activation of PPARα reduces insulin secretion from β-cells while promoting fatty acid oxidation in liver to limit steatosis and insulin resistance in response to increased fatty acid burden. CEACAM1 decreases fatty acid synthase activity (Najjar et al., 2005) to contribute to the regulation of malonyl-CoA conversion to palmitate and hence to determine its abundance relative to long chain fatty acyl CoA, a critical factor in fatty acid -oxidation. Thus, it is possible that the down-regulation of CEACAM1 by dietary fat constitutes a positive feedback mechanism on fatty acid -oxidation, not only by supplying newly synthesized fatty acids to activate PPARα (Chakravarthy et al., 2005) but also by removing the inhibitory effect of malonyl-CoA on fatty acid transport into the mitochondria (Patsouris et al., 2006, Durgan et al., 2006). This hypothesis is supported by the marked decrease of CEACAM1 level at fasting, an event that involves an accelerated shift from glycolytic to lipolytic metabolism, mediated by robust PPARα activation and removal of inhibition of carnitine palmitoyltransferase 1 by malonyl-CoA (Patsouris et al., 2006, Durgan et al., 2006).

Fibrates improve insulin sensitivity with a concomitant decrease in glucose-stimulated insulin secretion in diabetic patients (Ferrari et al., 1977). The mechanism underlying the
decrease in insulin secretion remains unclear, although Pparα activation of fatty acid oxidation in pancreatic β-cells and increased apoptosis in association with reduced triacylglycerol content have been proposed (Gremlich et al., 2005, Rubi et al., 2002, Liu et al., 2011, Cnop et al., 2005, Zhou et al., 1998). Under our experimental conditions, fenofibrate did not induce morphological change, nor did it affect intrinsic β-cell function in isolated islets. Instead, it appears to cause a decrease in insulin secretion as a consequence of its negative effect on hepatic insulin clearance. Mechanistically, this is mediated primarily by a PPARα-dependent down-regulation of CEACAM1 in liver. Together with normal insulin secretion in mice with null deletion of Ceacam1 (Cc1−/−) (DeAngelis et al., 2008), this assigns a key role for hepatic CEACAM1 in coordinating the regulation of lipid and insulin metabolism in liver to maintain systemic insulin sensitivity and glucose homeostasis.

The current studies demonstrated that the decrease in insulin secretion by fenofibrate is compensatory to reduction in insulin clearance in wild-type mice but not Pparα−/− mice, thereby maintaining normoinsulinemia and insulin sensitivity. Although reduced insulin clearance compensates for reduced insulin secretion in insulin-sensitive subjects (Rudovich et al., 2004), the study provides in vivo evidence that insulin secretion can also respond to changes in insulin clearance to maintain insulin and glucose homeostasis (Kim et al., 2016). Mechanistically, reduction of insulin secretion in response to decreased insulin clearance could be mediated by the negative feedback mechanism of acute rise of insulin resulting from reduced insulin clearance on insulin secretion from β-cells (Fig. 8). Collectively, this promotes changes in hepatic CEACAM1 levels and function as a
potential drug target against dyslipidemia in cardiometabolic diseases.
Figure Legends

FIGURE 1. Fatty acids down-regulate hepatic CEACAM1 expression in a PPARα-dependent manner. A, mice were fed RD or HF for 8 weeks, and liver lysates were subjected to Western blotting analysis of CEACAM1 (CC1) expression by immunoblotting the upper band with α-CC1 (upper part of the membrane) and α-tubulin antibody (lower part of the membrane), with the latter serving as a protein loading control. Gel represents more than two separate experiments of two mice/feeding group/experiment. B, panel i, quantitative RT-PCR analysis of Ceacam1 mRNA levels in primary hepatocytes extracted from WT and Pparα−/− mice and treated with 0.1 mM of individual or free fatty acid mixture (Mix) for 18 h. The values are expressed as means ± S.E. *, p <0.05 versus BSA (-)/genotype. Panel ii, Western blotting analysis of CEACAM1 in WT primary hepatocytes treated with 0.1 mM individual fatty acids for 0 –24 h (part a) and also in Pparα−/− treated with individual fatty acids and their mixture (Mix) for 24 h (part b). The gels were scanned, and the density of CEACAM1 bands relative to actin is represented in the graphs to the right. The values are expressed as means ± S.E. *, p <0.05 versus BSA-treated (-).

FIGURE 2. Differential metabolic effect of high fat diet in WT and Ppara−/− mice. A and B, male mice (n >6) were fed RD or HF for 7 weeks before intraperitoneal glucose tolerance (A) and insulin tolerance tests (B) were performed. C, acute phase insulin secretion in response to glucose was assessed in overnight fasted mice fed RD or HF for 8 weeks. The values are expressed as means ± S.E. *, p <0.05 HF- versus RD-fed WT; †, p <0.05 RD-fed Ppara−/− versus RD-fed WT; §, p <0.05 HF-fed Ppara−/− versus HF-fed.
FIGURE 3. **The PPARα agonist Wy14643 protects against diet-induced metabolic abnormalities.** Male mice were fed RD or HF for 9 weeks before supplementing the diet with 0.1% Wy14643 (Wy) to measure sequentially. *A*, glucose tolerance. *B*, insulin tolerance. *C*, after 10 days of treatment, livers were removed, and their lysates were subjected to Western blotting analysis of CEACAM1 protein levels, as in Fig. 1. *n* = 7–9 mice/genotype/feeding group. The values are expressed as means ± S.E. *, *p* < 0.05 HF-fed versus RD-fed/genotype; †, *p* < 0.05 Wy14643-supplemented HF versus HF/genotype.

FIGURE 4. **Fenofibrate reduces hepatic CEACAM1 protein levels.** Male mice (5 months old) were fed RD-supplemented with 0.1% fenofibrate (Feno) to measure sequentially. *A*, glucose tolerance. *B*, insulin tolerance. *C*, acute phase insulin secretion in response to glucose after 9, 13, and 20 days of treatment, respectively. *n* = 10 mice/feeding group/genotype. The values are expressed as means ± S.E. *, *p* < 0.05 fenofibrate versus RD. *D*, Western blotting analysis of CEACAM1 and CD36 in liver lysates from mice that had been fed for 3 weeks with fenofibrate-supplemented RD diet. The gel represents two separate experiments performed on more than four mice/group.

FIGURE 5. **Fenofibrate decreases insulin secretion in a non-cell autonomous manner.** *A* and *B*, male mice were fed a fenofibrate-supplemented RD diet for 4 weeks before pancreata were removed to carry out hematoxylin and eosin staining (*A*) and
assess insulin content by immunofluorescence analysis using polyclonal antibody against insulin (B). C, GSIS was performed in islets extracted from these mice in response to 2.8 and 16.8 mM glucose. Insulin level was normalized to the protein content of islet lysates. The experiments were repeated twice. The values are expressed as means ± S.E. *, p <0.05 16.8 versus 2.8 mM glucose.

FIGURE 6. Overexpressing CEACAM1 in liver prevents the negative effect of fenofibrate on insulin clearance and secretion. A, male WT and LCC1 mice (3 months of age) were fed a fenofibrate (Feno)-supplemented RD diet for 4 weeks before acute phase insulin release in response to glucose was assessed, and the area under the curve was measured and presented in the accompanying bar graph. B, mice were fasted overnight (until 1100 –1200 h the next morning) for blood drawing and assaying for plasma C-peptide (panel i) and insulin levels (panel ii) to calculate the C-peptide/insulin molar ratio as a measure of insulin clearance (panel iii). C, fasting blood glucose level was also assessed in these mice. The values are expressed as means ± S.E. *, p <0.05 versus RD/genotype; †, p <0.05 LCC1-Feno versus WT-Feno. D, the liver was extracted from some mice and lysed to assess protein level of CEACAM1 by Western blotting with rat (rCC1) and mouse (mCC1) antibodies. The lower part of each gel was immunoblotted with tubulin antibody to control for protein loading.

FIGURE 7. Fenofibrate decreases insulin secretion but improves insulin sensitivity in diet-induced obese mice. Male mice (3 months of age) were fed RD or HF for 9 weeks and treated with fenofibrate (Feno), as described in the legend of Fig. 4 to test. A,
glucose tolerance. B, insulin tolerance. C, acute phase insulin secretion in response to glucose. \( n = 6–9 \) mice/feeding group/ genotype. The values are expressed as means ± S.E. * \( p < 0.05 \) RD versus HF; † \( p < 0.05 \) RD versus RD-fenofibrate; § \( p < 0.05 \) RD versus HF-fenofibrate. D, Western blotting analysis was carried out to assess the combined effect of fenofibrate and HF on hepatic CEACAM1 protein levels. The gel represents two separate experiments performed on more than four mice/group.

FIGURE 8. Schematic diagram summarizing the effect of fenofibrate on insulin metabolism. Fenofibrate activates PPAR\( \alpha \) to reduce CEACAM1 expression in the liver and induce fatty acid oxidation (FAO). This causes an acute rise in insulin levels, which could in turn reduce insulin secretion to preserve physiologic insulin and glucose homeostasis.
References


Potential role of pp120/HA4, a substrate of the insulin receptor kinase. *J. Biol. Chem.* **270**, 24073-24077


Figure 1

A. Liver

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>RD</td>
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</tr>
<tr>
<td>α-CC1</td>
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<tr>
<td>α-Tubulin</td>
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B. Primary Hepatocytes

iia. WT (0-24 h)

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<tr>
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<tr>
<td>PA</td>
<td>0</td>
<td>6 12 24 h</td>
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<tr>
<td>α-CC1</td>
<td>1.5</td>
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<tr>
<td>α-Actin</td>
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<td>0.8</td>
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</table>

OA

α-CC1   | 1.2 |
α-Actin | 0.4 |

LA

α-CC1 (120 kDa) | 1.2 |
α-Actin (42 kDa) | 0.4 |

iiib. 24 h

WT

α-CC1

Pparaα/−

α-CC1

α-Actin
Figure 2

A. Glucose tolerance test

B. Insulin tolerance test

C. Acute-phase insulin secretion
Figure 3

A. Glucose tolerance test

WT

* †

Glucose (mg/dl)

Post-ip inject (min)

Ppara<sup>−/−</sup>

B. Insulin tolerance test

WT

* †

Blood glucose (% Basal)

Post-ip inject (min)

Ppara<sup>−/−</sup>

C. Western blot analysis

<table>
<thead>
<tr>
<th></th>
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<th>Ppara&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>RD</td>
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<td>HF-Wy</td>
</tr>
<tr>
<td>α-CC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>120 kDa</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 kDa</td>
</tr>
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</table>
Figure 4

A. Glucose tolerance test

![Glucose tolerance test graphs for WT and Pparα−/− mice.]

B. Insulin tolerance test

![Insulin tolerance test graphs for WT and Pparα−/− mice.]

C. Acute-phase insulin secretion

![Insulin secretion graphs for WT and Pparα−/− mice.]

D. Western blot

<table>
<thead>
<tr>
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<th>Pparα−/−</th>
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<tr>
<td>RD</td>
<td>Feno</td>
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</tr>
<tr>
<td>1</td>
<td>2</td>
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<tr>
<td>1</td>
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<td>1</td>
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- lb:α-CC1: 120 kDa
- relb:α-CD36: 78 kDa
- relb:α-GAPDH: 37 kDa
Figure 5

A. IHC

B. Immunostaining

C. GSIS in isolated islets

![Graph showing GSIS in isolated islets with different glucose concentrations and treatments.](image)
Figure 6

A. Glucose-stimulated insulin secretion

B. Insulin clearance

C. Fasting glucose

D. Hepatic CEACAM1 level

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<tr>
<th></th>
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<td></td>
<td>RD Feno</td>
<td>RD Feno</td>
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<tr>
<td>α-CC1</td>
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<tr>
<td>α-Tubulin</td>
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<td>50</td>
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<tr>
<td>α-mCC1</td>
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<td>120</td>
</tr>
<tr>
<td>α-Tubulin</td>
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<td>50 (kDa)</td>
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</table>
Figure 7

A. Glucose tolerance test

B. Insulin tolerance test

C. Acute-phase insulin secretion

D. Western blot analysis

<table>
<thead>
<tr>
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<th>WT</th>
<th>Pparα−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
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</tbody>
</table>

- α-CC1: 120 kDa
- α-Tubulin: 50 kDa
Figure 8

Fenofibrate

↑ PPAR\(\alpha\) activation

↓ CEACAM1

↓ Insulin clearance

↓ Acute increase in plasma insulin

↓ Insulin secretion

Normoinsulinemia

Normoglycemia
TABLE 1

Effect of Wy14643 treatment on plasma and tissue biochemistry in male mice

Male mice (n = 7–9/genotype/feeding group) were fed RD or HF diet for 9 weeks starting at 3 months of age. Diet was supplemented with Wy14643 for 10 days prior to sacrifice. The mice were fasted overnight (until 1100 –1200 h the next morning) for blood drawing and tissue extraction. Unless otherwise mentioned, the parameters are from plasma. The values are expressed as means ± S.E. HF-Wy, Wy14643-supplemented HF diet; BW, body weight; WAT, white adipose tissue; TG, triacylglycerol; C-peptide/insulin, steady state C-peptide/insulin molar ratio.

<table>
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<th>HF</th>
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<tbody>
<tr>
<td>BW (g)</td>
<td>27.3 ± 1.6</td>
<td>28.5 ± 1.1</td>
<td>31.7 ± 0.7</td>
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<tr>
<td>WAT/BW (%)</td>
<td>2.4 ± 0.3</td>
<td>2.6 ± 0.6</td>
<td>3.4 ± 0.5</td>
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<tr>
<td>Subcutaneous fat/BW (%)</td>
<td>2.5 ± 0.2</td>
<td>2.4 ± 0.6</td>
<td>3.8 ± 0.5</td>
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<tr>
<td>NEFA (mEq/liter)</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
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<tr>
<td>TG (mg/dl)</td>
<td>81.7 ± 3.6</td>
<td>79.4 ± 8.1</td>
<td>47.7 ± 8.6</td>
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<tr>
<td>Hepatic TG (μg/mg)</td>
<td>121.0 ± 1.0</td>
<td>119.0 ± 15.0</td>
<td>77.0 ± 15.0</td>
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<tr>
<td>Insulin (pM)</td>
<td>57.4 ± 1.3</td>
<td>56.0 ± 6.0</td>
<td>41.2 ± 2.4</td>
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<tr>
<td>C-peptide (pmol)</td>
<td>928.0 ± 84.0</td>
<td>531.0 ± 53.0</td>
<td>465.0 ± 58.0</td>
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<tr>
<td>Blood glucose (mg/dl)</td>
<td>15.9 ± 2.0</td>
<td>11.4 ± 0.3</td>
<td>15.7 ± 3.7</td>
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\( a \) \text{p < 0.05 versus RD/genotype.}\n
\( b \) \text{p <0.05 HF-Wy versus HF/genotype.}\n
\( c \) \text{p < 0.05 RD-fed Ppara \textsuperscript{-/-} versus RD-fed WT.}\n
212
TABLE 2

Effect of fenofibrate treatment on plasma and tissue biochemistry in male mice

Male mice (n = 6–9/genotype/feeding group) were fed RD or HF diet for 9 weeks starting at 3 months of age. The diet was supplemented with fenofibrate for 20 days prior to undergoing NMR analysis for body fat composition. Thereafter, the mice were fasted overnight (until 1100 – 1200 h the next morning) for blood drawing and tissue extraction. Unless otherwise mentioned, the parameters are from plasma. The values are expressed as means ± S.E. HF-Feno, fenofibrate-supplemented HF diet; BW, body weight; WAT, white adipose tissue; TG, triacylglycerol; C-peptide/insulin, steady state C-peptide/insulin molar ratio.

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<td>RD</td>
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<td>BW (g)</td>
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<td>Fat mass (%)</td>
<td>9.0 ± 1.2</td>
<td>20.0 ± 1.2</td>
<td>11.5 ± 1.2</td>
<td>16.0 ± 0.8</td>
<td>26.1 ± 0.7</td>
<td>21.4 ± 0.7</td>
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<td>Lean mass (%)</td>
<td>2.6 ± 0.5</td>
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<td>NEFA (mM/liter)</td>
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<td>1.3 ± 0.1</td>
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<td>1.1 ± 0.1</td>
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<td>TG (mg/dl)</td>
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<td>Hepatic TG (µg/mg)</td>
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<td>Insulin (pg)</td>
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<td>C-peptide (ng)</td>
<td>177.0 ± 7.0</td>
<td>240.0 ± 19.0</td>
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<td>C-peptide/insulin</td>
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</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>92.0 ± 4.0</td>
<td>98.0 ± 2.0</td>
<td>87.0 ± 2.0</td>
<td>92.0 ± 4.0</td>
<td>63.9 ± 4.0</td>
<td>57.3 ± 2.0</td>
</tr>
</tbody>
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a p < 0.05 versus RD/genotype.
b p < 0.05 HF-Feno versus HF/genotype.
c p < 0.05 RD-fed Ppara −/− versus RD-fed WT.
Chapter 7

Summary, Conclusion and Discussion

Our studies identify novel roles of CEACAM2 protein in regulation of insulin secretion, renal insulin clearance and subsequent insulin action. Previous studies have shown that CEACAM2 regulates insulin action by decreasing food intake and moderating energy expenditure (128, 132). Our recent findings show that global Ceacam2 knockout male mice (Cc2/−) have higher insulin secretion in response to glucose administration compared to their wildtype counterparts, supporting the fact that CEACAM2 inhibits insulin secretion. This is consistent with its higher expression during the fasting compared to the fed state and its function in regulating food intake (128). The identification of predominant CEACAM2 expression in alpha pancreatic cells compared to beta cells did not affect basal glucagon or somatostatin levels, indicating no antagonistic effect by these two latter hormones on insulin. Isolated pooled islets from mutant and wildtype mice did not show any difference in insulin release, suggesting an extrinsic pancreatic regulatory mechanism affecting insulin release through CEACAM2 deletion.

Consistent with CEACAM2 expression in the villi of distal intestinal regions where GLP-1 secreting-cells reside (37, 38), deletion of CEACAM2 caused a higher release of GLP-1. Given the importance of GLP-1 in stimulating insulin release from pancreatic beta
cells (39, 40), we showed that CEACAM2 effect on insulin release was partly through the GLP-1 pathway. Mutant mice revealed a marked increase of basal and oral glucose stimulated GLP-1 levels, further verifying this mechanism. Moreover, exendin (9-39), a GLP-1 receptor antagonist, abolished the effect of CEACAM2 on insulin secretion causing wildtype and mutant mice to release comparable levels of insulin in response to oral glucose administration. Similarly, studying GLP-1 release in GLUTag cells (42, 43), enteroendocrine L cells of the intestine, showed direct effect of CEACAM2 on GLP-1 release since silencing CEACAM2 by almost 50% was sufficient to rise GLP-1 levels upon glucose stimulation. The underlying mechanism was related to the increase of Ca^{2+} influx through L-type voltage dependent calcium channels (VDCC) of siRNA-mediated knockdown GLUTag cells compared to scrambled, consistent with the effect of CEACAM2 deletion on the increase in GLP-1 release. In summary, CEACAM2 has an indirect effect on regulating insulin release by reducing GLP-1 secretion from L cells and specifically lowering calcium entry through VDCC.

The following work also expanded research regarding CEACAM2 functions, including an innovative role in regulating renal insulin clearance. The fact that CEACAM1, a highly homologous protein of CEACAM2, regulates hepatic insulin endocytosis (98, 113) led us to investigate a possible role of CEACAM2 in renal insulin clearance since the kidney represents a main site of insulin disposal and degradation. Immunostaining showed that CEACAM2 is expressed in proximal tubule cells of the kidney cortex, the primary site for insulin disposal. Furthermore, the long isoform of CEACAM2 (CEACAM2-2L) is highly expressed in proximal tubule cells indicating the presence of a CEACAM2 active form that harbors important phosphorylation sites necessary for
insulin action. In kidney lysates, CEACAM2 was shown to be an insulin receptor substrate, as supported by its phosphorylation upon insulin stimulation at its intra cellular phosphorylation sites promoting receptor mediated endocytosis. By using a mouse model with global deletion of CEACAM2, we found that mutant mice have insulin clearance dysfunction compared to wildtype, given that internalization of $I^{125}$ labeled insulin in proximal tubule cells was diminished. This was further supported by higher expression of insulin receptor alpha biotinylated proteins on the surface membrane of isolated proximal tubule cells from mutants upon insulin stimulation, indicating lower internalization and insulin extraction in the kidney.

Previous studies on young male Ceacam2 knockout mice did not exhibit obesity and insulin resistance despite their state of hyperphagia, owing to the hypermetabolism state and increased energy expenditure (132). This led to insulin sensitivity and normal plasma insulin levels. Despite the elevated insulin secretion in Cc2$^{-/-}$ mice, they were insulin sensitive owing to increased C-peptide to insulin (C/I) molar ratio, an indicator of insulin clearance, which was consistent with high hepatic Ceacam1 levels being an important factor in regulating hepatic insulin clearance and enhancing insulin sensitivity (98, 113). Insulin resistance developed in mutant mice starting at about 9 months of age. It was caused by hyperphagia, reduced sympathetic outflow to white adipose tissue, reduced energy expenditure in skeletal muscle, and impaired insulin clearance as assessed by insulin and glucose intolerance, hyperinsulinemia, and decline in hepatic insulin signaling. The age dependent insulin resistance in older mutants was accompanied by a decline in hepatic Ceacam1 expression that was more significant than the decrease shown in the wildtype. This finding shows that age mediates the reduction of hepatic Ceacam1
expression. In addition, hyperphagia induced obesity and hyperinsulinemia decrease Ceacam1 expression in mutants more dramatically than in wild type counterparts. Findings in 12-month old Cc2/− mice also revealed that hyperinsulinemia over time did not affect either alpha or beta islet cell area relative to total pancreas, despite the increase in acute phase of insulin secretion in response to glucose administration at all age groups. This reflects intact and functional beta pancreatic cells that prevent the initiation of any fasting hyperglycemia signs.

These studies provide novel evidence of the role of CEACAM2 protein in regulating insulin secretion and renal insulin clearance to maintain normal plasma insulin levels and induce insulin sensitivity. Reduced hepatic CEACAM1 levels with aging mediates impairment of insulin clearance and hyperinsulinemia, causing insulin resistance in global CEACAM2 knockout mice. Further studies are needed to investigate the role of CEACAM1 decrease in expression with age. These studies expand our knowledge proposing that CEACAM proteins could be potential targets for metabolic syndrome abnormalities.
References


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