A Dissertation

entitled

A Role for CEACAM2 in Insulin Homeostasis and Action

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for
the Doctor of Philosophy Degree in Biomedical Sciences

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August 2010
An Abstract of

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July 2010

The carcinoembryonic antigen related cell adhesion molecule (CEACAM) family of proteins is involved in insulin signaling, lipogenesis, immunity, and tumor suppression. To identify a function for CEACAM2, we created a murine model without expression of the Ceacam2 gene. We report that the male Ceacam2 knockout mice are overall insulin sensitive. This occurs in light of hyperphagia and insulin resistance in skeletal muscle. Sensitivity is likely due to increased energy expenditure and to higher rates of insulin secretion. CEACAM2 localization to the ventromedial hypothalamus suggests a role for CEACAM2 in central regulation of energy balance and peripheral insulin action and secretion.
To the people who taught me first, and from whom I continue to learn each day.

Your boundless strength supports me and helps me overcome obstacles far less daunting than the ones you have faced fearlessly. Your overwhelming generosity—for you always give in any and every way you can—sustains me.

To the people to matter most, Aruna and Ramesh Patel.

I could not have done it without you.

This accomplishment is yours as much as it is mine.

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Acknowledgements

I have had the good fortune of pursuing my doctoral research under the mentorship of Dr. Sonia M. Najjar. Your zest for understanding is contagious, and you have demonstrated time and again that one must never be lax in the pursuit of excellence. Science (and life!) will unfold as it will, so we must remain open to new directions. A sharp eye and a perked ear are essential, because opportunities are everywhere. You saw my potential and you fostered it, so that today I am a scientist; but most incredibly you made me believe in me too. Thank you for every moment you spent with me.

This dissertation would not have been possible without the love and support I received from my parents and my sisters: Ramesh and Aruna and Hetal, Tejal and Monal Patel. All the good that resides in me originated in you. Your strong faith; your dedication to work; your ability to stay grounded when it seems everything is up in the air. You gave me exactly what I needed, whether it was a hug or a voice of reason or a theme song or a swift kick or a pile of hahahahe. You are all a part of this. Thank you. A million times over.

A humongous thank you also to many friends, especially my dearest friend, Veena. You have been through it all with me, and your encouragement, like your smile, never wavered. You are one my blessings, and I am so grateful.
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INTRODUCTION

The National Health and Nutrition Examination Survey conducted by the National Center for Disease Statistics, Centers for Disease Control and Prevention provides the opportunity to track obesity trends in the United States. Comparison of data from 1976-1980 with that from 2007-2008 has shown that the prevalence of obesity in adults of ≥20 years of age has more than doubled, going from 14.5% to 33.8% (Flegal, Carroll et al.; Flegal, Carroll et al. 1998). Obesity is defined as body mass index (BMI) of 30.0 or higher, as calculated by mass in kilograms divided by height in meters squared. Obesity has been directly associated with higher risk factors, including hypertension, dyslipidemia and type 2 diabetes mellitus (T2DM) (Malnick and Knobler 2006).

Cardiovascular disease and T2DM are integral components of the metabolic syndrome, a cluster of abnormalities that are commonly associated with insulin resistance (Wilson, D'Agostino et al. 2005). Poor insulin-stimulated glucose uptake and homeostasis is a common phenomenon in the majority of patients with impaired glucose tolerance and T2DM, as well as in ~25% of non-obese individuals with normal glucose tolerance (Reaven 1988). Insulin-resistant individuals with normal glucose tolerance are able to overcome the defect in insulin action through enhanced insulin secretion when compared with a control insulin-sensitive group (Hollenbeck and Reaven 1987). Frank diabetes and
pathophysiological consequences of resistance to insulin-mediated glucose disposal manifest in individuals who are no longer able to sustain compensatory hyperinsulinemia (Reaven 1988).

**Insulin**

The peptide hormone insulin is synthesized in pancreatic β-cells. As an anabolic hormone, insulin regulates several cellular processes including cell growth and proliferation. The primary function of insulin is to maintain plasma glucose level within a very narrow range, particularly following a meal (Tirone and Brunicardi 2001; Whittaker and Whittaker 2005).

Glucose, therefore, plays a pivotal role in the control of insulin production. Glucose uptake and metabolism acts as a potent transcriptional activator of the insulin gene (German 1993), which in humans is located on chromosome 11p15.5 (Harper, Ullrich et al. 1981). Insulin gene transcription is tightly regulated (Laub and Rutter 1983), and defects lead to several types of diabetes, in particular the Mature-Onset Diabetes of the Youth (MODY) family of diseases (Kulkarni and Kahn 2004).

The initial transcript of the insulin gene yields preproinsulin mRNA. As preproinsulin polypeptide chain is translated, a signaling sequence targets its translocation to the intracisternal space of the rough endoplasmic reticulum (Eskridge and Shields 1983). In this compartment, preproinsulin polypeptide
undergoes proteolytic cleavage to yield proinsulin polypeptide (Lively 1989), consisting of A and B chains linked to a connecting peptide (C-peptide) by adjacent pairs of basic residues (Steiner, Chan et al. 1985). Proinsulin polypeptide undergoes folding to allow for the formation of two disulfide bridges between the A and B chains, prior to being packaged into secretory granules (Rhodes, Lucas et al. 1987). Proteolytic cleavage of proinsulin molecules inside these clathrin-coated granules liberates biologically active insulin from C-peptide (Orci, Ravazzola et al. 1987; Rhodes and Alarcon 1994). Mature 5.8 kDa insulin proteins and C-peptides reside in β-cell granules until they are ready to be released at a molar ratio of 1 in response to stimuli.

**Insulin Secretion**

Tight regulation of insulin secretion with glucose uptake is possible because β-cells are electrically excitable and produce action potentials in response to glucose metabolism (Dean and Matthews 1968; Dean and Matthews 1970).

Glucose enters β-cells by facilitated diffusion through GLUT2 transporters and is captured within the β-cell via phosphorylation by glucokinase (Guillam, Dupraz et al. 2000). As glucose-6-phosphate undergoes glycolysis, ATP is produced, elevating the intracellular ATP/ADP ratio. Increased levels of ATP act on ATP-sensitive potassium channels, binding and causing closure (Seino,
Iwanaga et al. 2000), and subsequently, membrane depolarization. This results in the opening of voltage-gated Ca\(^{2+}\) channels and Ca\(^{2+}\) influx (Wollheim, Janjic et al. 1981), which triggers exocytosis of insulin-loaded granules into the portal circulation (Kibbey, Pongratz et al. 2007).

An abundance of such granules are present in \(\beta\)-cells, either in the cell interior or docked at the plasma membrane (Straub and Sharp 2004). Subcellular localization determines availability of the granules for exocytosis. Those within the cellular interior form the reserve pool. The granules located at the plasma membrane that are primed and capable of release make up the readily releasable pool (RRP). These distinct pools provide the basis for biphasic insulin secretion in response to glucose (Bratanova-Tochkova, Cheng et al. 2002).

The first phase of insulin release consists of approximately 50 RRP granules that are located proximally to the voltage-gated Ca\(^{2+}\) channels (Barg, Eliasson et al. 2002). Exocytosis of insulin occurs within 100-200ms of Ca\(^{2+}\) channel activation, and insulin level peaks 5 minutes after glucose stimulus (Barg, Eliasson et al. 2002; Bratanova-Tochkova, Cheng et al. 2002).

Whereas the insulin level during first phase of release is characterized by a peak, the second phase is an elevated plateau. Second-phase insulin secretion relies on the capacity of the \(\beta\)-cell to mobilize and biochemically alter the reserve pool of granules to recruit to the RRP and continue exocytosis (Barg, Eliasson et al. 2002; Bratanova-Tochkova, Cheng et al. 2002). Evidence suggests that the rate
of RRP refilling exceeds the rate of exocytosis, so the RRP may be replenished (Bratanova-Tochkova, Cheng et al. 2002). Secretion continues for several minutes at a steady rate (Barg, Eliasson et al. 2002), and in normal β-cells this is sustained until the glucose stimulus is relieved (Kulkarni and Kahn 2004).

Pancreatic β-cells secrete insulin in response to other nutrients in addition to glucose. Some of these, like glucose, are initiators of release, whereas others, like arginine, act as potentiators of release induced by glucose (Smith, Sakura et al. 1997). Arginine stimulates secretion in the presence, but not absence, of glucose (Hermans, Schmeer et al. 1987) by direct depolarization of the β-cell (Smith, Sakura et al. 1997). Arginine accumulation in the β-cell produces the insulinotrophic effect, as arginine does not increase the energy state of β-cells through catabolism of arginine itself or of glucose (Blachier, Leclercq-Meyer et al. 1989; Thams and Capito 1999). Rather, transport of the positively-charged amino acid raises the membrane potential, stimulating voltage-gated Ca\(^{2+}\) entry, with a subsequent rise in intracellular Ca\(^{2+}\) concentration and insulin secretion (Smith, Sakura et al. 1997).

**Insulin Receptor**

Secreted insulin mediates its plasma glucose-lowering action by binding to and activating a specific plasma membrane receptor. Insulin receptor (IR) signaling regulates many cellular processes, including glucose transport, cell
growth, and synthesis of glycogen, proteins and fatty acids. While the IR gene is transcribed and translated in the majority of tissues, it is most highly expressed in insulin target tissues, including adipose, brain, liver and muscle.

The 22 exons encode the extracellular α subunit, which contains the insulin-binding region, and the β subunit, which is made up of an extracellular domain, a single transmembrane helix and the cytosolic tyrosine kinase domain (Seino, Seino et al. 1989). The active site catalytic loop hydrolyzes ATP leading to phosphorylation of cytoplasmic tyrosine residues (Hubbard, Wei et al. 1994). This function classifies IR as a member of the receptor tyrosine kinase family.

Functional insulin receptor is composed of two α and two β subunits, linked by disulfide bridges into a tetramer that is essentially a dimeric protein complex (Lee and Pilch 1994). Two molecules of insulin are able to bind to the IR dimer (Whittaker and Whittaker 2005), inducing a conformational change in the β subunit from cis-inhibition of the catalytic loop to trans-activation by autophosphorylation of tyrosine kinase residues (Hubbard, Wei et al. 1994). For example, phosphorylated tyrsoine 960 acts as a docking site for the phosphotyrosine binding of IR substrates, such as Src homology 2 domain containing (SHC) protein and the insulin receptor substrate (IRS) proteins (Paz, Voliovitch et al. 1996). Phosphorylation of these substrates activates a cascade of downstream signaling pathways that ultimately mediate insulin actions.
Insulin Signaling Pathways

The insulin signaling cascade is complex and involves activation of multiple pathways to mediate the metabolic and mitogenic actions of insulin.

The major substrates of IR are insulin receptor substrate (IRS) proteins. Activated IRS phosphorylates the Src homology 2 domains of proteins to activate pathways leading to glucose transport (White and Kahn 1994). Phosphorylation of IRS proteins propagates the metabolic actions of insulin through the phosphatidylinositol 3-kinase (PI3K) pathway (Sun, Rothenberg et al. 1991). PI3K pathway stimulation leads to mobilization of glucose transporters, an increase in glycolysis, glycogen synthesis, protein synthesis, and inhibition of apoptosis (Shepherd, Withers et al. 1998).

Another important substrate of IR is Src homology 2 containing (SHC) protein, which mediates mitogenic activity of insulin via the RAS/RAF/MAPK pathway (Pronk, McGlade et al. 1993). Activation of this cascade by several growth factors induces gene transcription for cell growth. However insulin is a less potent activator of the MAPK pathway than other signaling molecules, such as epidermal growth factor and platelet derived growth factor (Yokote, Mori et al. 1994). SHC also plays a role in receptor-mediated endocytosis of insulin via clathrin coated pits (Okabayashi, Sugimoto et al. 1996). Importantly, IR stimulation of the MAPK pathway is unaffected by the development and onset of
insulin resistance; the PI3K pathway, on the other hand, is blunted in the face of insulin resistance.

**Physiological Actions of Insulin in Muscle and Adipose**

The binding of insulin to its receptor is the initiating event to stimulate several cellular processes. Because insulin-mediated pathways are propagated by downstream substrates, and because substrate expression varies from cell type to cell type, the pathways evoked by insulin are tissue-specific. Substrate expression, then, defines the response to insulin.

Skeletal muscle is the major site for post-prandial insulin-stimulated glucose disposal. Muscle internalizes glucose and oxidizes it for energy or stores it as glycogen following uptake (Shearer and Graham 2002), as insulin increases the activity of glycogen synthase (Rossetti and Giaccari 1990). Insulin binds to and activates IR leading to tyrosine phosphorylation of IRS-1 to recruit PI3K, which, through PIP3 activation of PDK1 and mTOR, triggers Akt2-mediated translocation of GLUT4 glucose transporter protein to the plasma membrane (Huang and Czech 2007). Glucose influx rapidly follows (Bouche, Serdy et al. 2004). GLUT4 thus is a key regulator in whole-body glucose homeostasis.

GLUT4 is also the major isoform of sugar transporter in adipose tissue (Huang and Czech 2007), where glucose is ultimately stored as triglyceride. As glucose is taken up and metabolized, malonyl-CoA level increases. Malonyl-CoA...
CoA inhibits the action of carnitine palmitoyl transferase 1 (CPT1), which provides the rate-limiting step for fatty acid oxidation in the mitochondria. Therefore insulin action elevates malonyl-CoA level, suppressing fatty acid oxidation, and shunts fatty acid toward production of triglyceride (McGarry and Foster 1980). It is important to note that adipocytes also remove triglycerides from plasma in response to insulin through activation of lipoprotein lipase (Mead, Irvine et al. 2002).

**Physiological Actions of Insulin in Liver**

The liver expresses a different isoform of glucose transporter protein, GLUT2 (Fukumoto, Seino et al. 1988). GLUT2 facilitates bidirectional glucose transport: it takes up glucose from circulation in the absorptive state, and in fasting conditions GLUT2 releases glucose into the blood (Leturque, Brot-Laroche et al. 2009). The liver, then, senses dietary sugar availability and builds or mobilizes glucose stores accordingly through GLUT2.

The liver integrates several metabolic processes in order to maintain whole-body glucose homeostasis. In the postabsorptive, or fasting, state hepatocytes produce glucose via glycogenolysis or gluconeogenesis. Upon feeding insulin potentiates a sharp decrease in hepatic glucose output through inhibition of glycogen phosphatase, the enzyme which catalyzes glycogen breakdown into glucose (Hother-Nielsen, Henriksen et al. 1996). Insulin
signaling phosphorylates forkhead transcription factor protien 1 (Foxo1) via protein kinase B (Guo, Rena et al. 1999). Phosphorylation of Foxo1 disrupts transactivation of gluconeogenic genes, such as glucose-6-phosphatase (Schmoll, Walker et al. 2000), and leads to nuclear exclusion of Foxo1 (Scheimann, Durham et al. 2001). Thus, in response to insulin, hepatocytes switch from processes that generate glucose to those that store glucose as glycogen or metabolize it into lipids (Leturque, Brot-Laroche et al. 2009). Whole-body glucose homeostasis is achieved in large part by tight regulation of hepatic glucose production (HGP).

**Insulin Level Mediates Insulin Action**

HGP modulation by insulin is sensitive to changes in portal vein (hepatic sinusoidal) insulin concentration (Sindelar, Chu et al. 1998). A two-fold increase in hepatic sinusoidal insulin causes near-complete inhibition of net hepatic glycogenolysis (Edgerton, Cardin et al. 2001).

Indeed, anatomical organization obligates the liver to maintain sensitivity to insulin level. Upon its release from beta-cells, insulin first traverses the liver before its delivery to the systemic circulation (Field 1973). Thus the liver is exposed to concentrations of insulin two to three times the level in the arterial blood. Hepatocytes extract ~70% of endogenous insulin during the basal state (Toffolo, Campioni et al. 2006) and rapidly adjust clearance rate when insulin pulses are secreted in response to nutrient (Meier, Veldhuis et al. 2005). Hepatic
insulin extraction, therefore, plays an important role in regulating peripheral insulin concentrations (Field 1973). Insulin degradation follows receptor-mediated endocytosis (Terris and Steiner 1975), and our lab has shown that CEACAM1 plays a pivotal role in promoting insulin clearance.

**CEACAM1**

Carcino Embryonic Antigen-related Cell Adhesion Molecule 1 (CEACAM1) is a highly conserved transmembrane glycoprotein. It is encoded by a single gene (*Ceacam1*) in the rat and human genome (Cheung, Thompson et al. 1993; Prall, Nollau et al. 1996). *Ceacam1* transcripts are ubiquitously expressed with a predominant distribution in liver. *Ceacam1* gene contains a functional promoter and nine exons (Najjar, Accili et al. 1993). During mRNA processing, exon 7 is alternatively spliced which results in the expression of two isoforms, the short isoform (CEACAM1-4S) lacking 61 of the 71 amino acids of the intracellular domain and the long isoform (CEACAM1-4L) which includes serine (Ser^{503}) and tyrosine (Tyr^{488}) phosphorylation sites (Lin, Luo et al. 1995). The short isoform lacks the intracellular phosphorylation sites of the 4L isoform (Lin, Luo et al. 1995). CEACAM1 mediates various functions, including cell adhesion (Obrink 1997) and tumor suppression (Luo, Earley et al. 1998), and acts as a
mediator of angiogenesis (Liu, Docherty et al. 2002) and T-lymphocyte signaling (Nagaishi, Pao et al. 2006).

**Regulation of CEACAM1 Phosphorylation:**

CEACAM1 is a substrate of both the insulin receptor (IR) (Najjar, Philippe et al. 1995) and the epidermal growth factor receptor (Abou-Rjaily, Lee et al. 2004). IR tyrosine kinase-mediated phosphorylation of CEACAM1 occurs in hepatocytes, but not in muscle or adipose tissues (Najjar, Philippe et al. 1995). In response to insulin, phosphorylation of Tyr\textsuperscript{1316} on IR indirectly phosphorylates Tyr\textsuperscript{488} on the intracellular region of CEACAM1 (Soni, Lakkis et al. 2000). Ser\textsuperscript{503} must be active (phosphorylated) for CEACAM1 to mediate response to insulin (Najjar, Philippe et al. 1995). SHP-1 and SHP-2 phosphatases dephosphorylate CEACAM1 (Huber, Izzi et al. 1999).

**Regulation of Insulin Action by CEACAM1:**

CEACAM1 regulates insulin action by promoting insulin clearance (Poy, Yang et al. 2002; Dubois, Bergeron et al. 2006) and de novo lipogenesis in liver (Najjar, Yang et al. 2005).

**Regulation of insulin clearance by CEACAM1:** Insulin clearance, occurring mostly in liver (Duckworth, Bennett et al. 1998), is mediated by the delivery of insulin via its receptor followed by its degradation in the hepatocyte (Terris and Steiner 1975; Sonne 1988). Upon phosphorylation by IR, Tyr\textsuperscript{488} on CEACAM1
binds to the SH2 domain of Shc, another substrate of IR (Poy, Ruch et al. 2002). Formation of the IR/CEACAM1/Shc complex in clathrin-coated vesicles (Rapoport, Miyazaki et al. 1997) allows for degradation of insulin (Formisano, Najjar et al. 1995) in the lysosome and recycling of IR to the plasma membrane (Sato, Terasaki et al. 1991). Co-expressing CEACAM1-4L, but not –4S and its site-directed phosphorylation-defective Ser\(^{503}\) to Ala (S503A) and Tyr\(^{488}\) to Phe (Y488F) mutants, increases receptor-mediated insulin endocytosis and degradation in NIH 3T3 cells (Formisano, Najjar et al. 1995; Najjar, Choice et al. 1998).

Consistent with the hypothesis that CEACAM1 promotes insulin degradation, the L-SACC1 mouse with liver-specific overexpression of the dominant negative phosphorylation-defective S503A CEACAM1 mutant developed hyperinsulinemia resulting from impaired insulin clearance (Poy, Yang et al. 2002). Hyperinsulinemia caused secondary insulin resistance (Park, Cho et al. 2006). The L-SACC1 mouse also developed visceral adiposity with elevated plasma FFA and plasma and hepatic triglyceride levels.

As in the L-SACC1 mouse, mice with global null mutation of Ceacam1 (Cc1\(^{-/-}\) mice) exhibited impairment of insulin clearance and hyperinsulinemia, elevated serum and hepatic triglyceride content, and heavier body mass (DeAngelis, Heinrich et al. 2008), suggesting that the primary role for CEACAM1 in insulin action is the promotion of insulin clearance in the liver.
A role of CEACAM1 in lipogenesis in liver: Fatty acid synthase (FAS), a key enzyme in the de novo synthesis of fatty acids, converts malonyl-CoA to palmitate, a saturated fatty acid. FAS plays an important role in regulating the level of malonyl-CoA, a critical fuel sensor that applies an allosteric inhibition of carnitine palmitoyltransferase 1 (CPT-1), the main enzyme catalyzing the transport of long chain fatty acids into mitochondria for oxidation.

FAS level is regulated by insulin. Chronically elevated levels of insulin, as in hyperinsulinemia and obesity, increase mRNA levels of Fasn (the gene that encodes FAS) and other lipogenic enzymes to promote lipogenesis, as supported by elevated hepatic FAS levels in obese rats (Bazin and Lavau 1982; Shillabeer, Hornford et al. 1992). In contrast to its long-term positive effect on FAS, evidence has been presented that insulin acutely decreases FAS activity in liver, but not in adipose tissue (Najjar, Yang et al. 2005). The decrease in FAS activity depends on the ability of insulin to induce CEACAM1 phosphorylation and internalization as part of insulin endocytosis and binding to FAS. Furthermore, this effect depends on the prior insulinemic state. For instance, insulin did not reduce FAS activity in obese hyperinsulinemic mice (L-SACC1 and Ob/Ob). This is consistent with blunted ability of insulin to activate insulin receptors and phosphorylate CEACAM1 in chronic hyperinsulinemia.
It is proposed that the acute negative effect of insulin on FAS activity occurs to limit lipogenesis in liver, which could develop in the presence of chronically high levels of insulin in the portal circulation (Ward, Walters et al. 1990). In this sense acute downregulation of FAS activity by insulin mediates its negative effect on hepatic triglyceride synthesis and output (Lewis and Steiner 1996; Malmstrom, Packard et al. 1998), and constitutes a mechanism to maintain insulin sensitivity in liver. This novel finding places CEACAM1 at the crossroads of altered fat and insulin metabolism in the pathogenesis of obesity and type 2 diabetes.

The role of CEACAM1 in the intertwined paths of insulin and fat metabolism: The L-SACC1 and the Cc1/- mouse phenotypes demonstrate a primary role of insulin clearance in maintaining insulin action in lipid metabolism (Bergman and Ader 2000). For instance, it reveals that impaired insulin clearance causes hyperinsulinemia and subsequently, hepatic insulin resistance, as assessed by reduced ability of insulin to suppress gluconeogenesis in hyperinsulinemic-euglycemic clamp studies (Park, Cho et al. 2006), and increased hepatic de novo synthesis and secretion of triglyceride as very low-density lipoproteins (VLDL). Increased VLDL-TG levels trigger compensatory insulin secretion to sensitize adipose tissue and muscle to hydrolyze TG and store its product. This increases adipocyte proliferation and adipose mass (visceral obesity), and decreases glucose uptake in muscle and adipose tissue. CEACAM1 constitutes a
mechanism by which insulin signaling modulates insulin sensitivity through regulating hepatic insulin clearance.

**CEACAM2**

While *Ceacam1* and its various functions have been extensively studied, little is understood about a highly related gene, *Ceacam2*. *Ceacam2* was first identified in BALB/c spleen and SJL/J liver (Nedellec, Dveksler et al. 1994). The gene was first called biliary glycoprotein 2 (*Bgp2*) due to its high similarity to *Bgp1* (now called *Ceacam1*) (Nedellec, Dveksler et al. 1994). The genes are both organized into 9 exons and 8 introns, and they share 79.6% sequence homology, with most differences occurring as nucleotide substitutions. Large intronic insertions occur in introns 2, 5 and 7 (Han, Phan et al. 2001). The functions of the gene products, however, are dissimilar: CEACAM2 does not act as a cell adhesion molecule as CEACAM1 does (Robitaille, Izzi et al. 1999), despite expressing an identical intracellular region (Han, Phan et al. 2001).

Evidence exists for differential function of CEACAM proteins. In terms of tissue distribution, *Ceacam1* is fairly ubiquitously expressed, found in liver, pancreas, kidney, endometrium, epithelial cells of the intestine, and hematopoietic cells (Thompson 1995; Hammarstrom 1999). *Ceacam2*, on the other hand, possesses more limited expression, confined to kidney, spleen, testis,
prostrate and brain (Zebhauser, Kammerer et al. 2005). Metabolic state appears to regulate CEACAM protein expression. Hepatic CEACAM1 is low at fasting, and rises with post-prandial peaks in serum insulin. The opposite pattern is observed in brain CEACAM2 expression, which is highest at fasting and low when insulin is elevated (Heinrich, Ghosh et al.). Because tissue distribution and metabolic expression pattern is unique from Ceacam1, the function of Ceacam2 gene product may also be distinct.

Null mutation of Ceacam2 yields a mouse model (Cc2/) in which females, but not males, display obesity, which results from hyperphagia and reduced energy expenditure (Heinrich, Ghosh et al.). Hyperphagia leads to peripheral insulin resistance, particularly in skeletal muscle (Heinrich, Ghosh et al.). Cc2/ female mice exhibit hyperinsulinemia, based on increased insulin secretion (Heinrich, Ghosh et al.). Here the elevated insulin levels act on hypothalamic fatty acid synthase to increase its activity, and this may contribute to hyperphagia (Heinrich, Ghosh et al.). Observations in the Cc2/ female mice point toward a role for CEACAM2 in the ventromedial hypothalamus, an area of central control of food intake and energy expenditure.

**STUDY OBJECTIVES:**

The observation that unlike female mice, the age-dependent gain of body mass was not altered in Cc2/ males and that their total fat mass was reduced
(Heinrich, Ghosh, et al.) provided the impetus to investigate the underlying mechanisms. To this end, we began by fully characterizing the metabolic phenotype in male animals. To further elucidate the mechanisms underlying the sexual dimorphism in the phenotype of Ceacam2 null mice, we will investigate the effect of gonadectomy on insulin action and secretion, and on energy balance.
RESULTS

Generation of Cc2<sup>−/−</sup> Null Mice

A 345 bp Xba I-Sac I fragment containing exon 1 and flanking regions in the promoter and the proximal region of intron 1, was replaced with sequences derived from plasmid pGK neo<sup>r</sup> (Fig. 1A). The Apa I-linearized targeting vector was electroporated into J-1 ES cells. Southern blotting was performed to screen ES clones. As expected, digestion of genomic DNA from wild-type and recombinant allele with Xba 1/Nsi 1 produced a 6.7-kb and 8.2-kb fragment, respectively, and positive ES cell clones out of 200 were identified (Fig. 1B). ES cells positive for the targeting event were injected into mouse blastocysts. Two resultant chimeric mice were bred with C57BL/6 mice (BL6), and Cc2<sup>+/−</sup> mice were identified by a Polymerase Chain Reaction (PCR) using gDNA as template and as indicated in Fig. 1A, an antisense primer from exon 2 (α-123) with a sense primer from exon 1 (S-107) to detect the wild-type sequence or a sense primer from the Neo<sup>r</sup> cassette (S-Neo<sup>r</sup>) to detect the knockout sequence (not shown). Heterozygous mice were mated to produce wild-type (WT) and Cc2<sup>−/−</sup> homozygous mice, identified by the 950 bp and the 1.9 kb PCR products, respectively, using the primer sets described above (Fig. 1C). Of note, the Ceacam1 allele remained intact in genomic isolates from both Cc2<sup>+/+</sup> and Cc2<sup>−/−</sup> mice (Fig. 1C).
To confirm the deletion of Cc2 gene, we performed RT-PCR using mRNA from the liver of WT, Cc2+/–, and Cc2−/– mice (Fig. 2A). A 50% and 100% loss of Cc2 cDNA content was detected in Cc2 heterozygous and homozygous mice, respectively (Fig. 2A). The specificity of the probes was examined by DNA blotting and hybridizing with γ32P ATP-labeled Cc2- specific probe (Fig. 2Aii). Using primers specific to either Ceacam1 or Ceacam2, RT-PCR detected Ceacam1 mRNA in isolated pancreatic islets from both Cc2+/– and Cc2−/– mice, as opposed to Ceacam2 mRNA, which was lost in Cc2−/– mice (Fig. 2B).

Deletion of the Cc2 gene in homozygous mice was also shown by total loss of Cc2 mRNA expression in the kidney, as detected by Northern analyses (not shown). Western analysis of total lysates following immunoprecipitation with an anti-CEACAM1 antibody revealed intact CEACAM1 protein levels in kidney (Fig. 2C, i) and liver (Fig. 2C, ii) of Cc2−/– mice.

**Body Composition of Male Cc2−/– Mice**

Unlike female Cc2−/– mice, which gain significantly higher body mass than their Cc2+/– counterparts, male Cc2−/– animals maintain a body mass similar to that of Cc2+/– mice throughout their lifespan (Heinrich, Ghosh et al.). 1H-MRS imaging reveals that male Cc2−/– mice have similar lean mass but reduced fat mass compared to Cc2+/– (Fig. 3). Moreover, the visceral adipose depot and adipocyte size are comparable in both groups of male mice at 3 months of age.
(not shown). Consistently, serum leptin levels are normal in male $Cc2^{-/-}$ mice at 3 months of age (Table).

**Elevated Insulin Secretion in Male $Cc2^{-/-}$ Mice**

Having shown that CEACAM2 is expressed in the ventromedial hypothalamus (VMH) (Heinrich, Ghosh et al.), a major glucosensing center in the brain (Silver and Erecinska 1998), we then investigated whether $Ceacam2$ deletion modulates insulin secretion. 3 month-old male $Cc2^{-/-}$ mice exhibit normal fasting insulin levels compared to $Cc2^{+/+}$ controls (Table). The significant increase in steady state C-peptide level (Table) suggests a greater rate of insulin secretion in these mice. However, this does not lead to hyperinsulinemia given the compensatory increase in insulin clearance, as suggested by elevated C-peptide/Insulin molar ratio in $Cc2^{-/-}$ mice (Table). The increase in insulin clearance is most likely due to intact levels of CEACAM1, a protein that promotes insulin clearance in liver (Fig. 2C).

Immunohistochemical analysis (IHC) localizes CEACAM2 to pancreatic α-cells (Fig. 4). Serum glucagon level one hour following insulin injection is intact in $Cc2^{-/-}$ mice. Basal serum somatostatin level is also normal following an overnight fast (Table). This suggests that the increase in insulin secretion is not due to altered release of its antagonists, glucagon and somatostatin.
Beta-cell Function is Enhanced in Male Cc2–/– Mice

To further assess insulin secretion in these mice, we determined insulin release in response to a glucose challenge or L-arginine (Fig. 5A) in overnight fasted mice. Acute-phase insulin release in response to glucose (2.0g/kg) is higher in 6 month-old Cc2–/– than control mice (Fig. 5Ai). Similarly, L-arginine (0.5 g/kg), a secretagogue that acts on the K+ channel to cause membrane depolarization independently of glucose, induces a larger rise in insulin secretion in 6 month-old Cc2–/– than control mice (Fig. 5Aii). Enhanced insulin secretion in the knockout mouse occurs in spite of lower β-cell mass when compared to wild-type, as shown by histomorphometry analysis (Fig. 5B). This supports enhanced β-cell function in Cc2–/– mice.

To further evaluate β-cell function in vivo, we subjected mice to hyperglycemic clamp analysis. Plasma glucose levels were clamped at 300 mg/dL during the clamp period (Fig. 6A). This was maintained by constant glucose infusion rate (Fig. 6B). During the clamp period, Cc2–/– mice had higher insulin levels at the same plasma glucose concentration (Fig 6C), indicating a higher rate of secretion than wild-type in response to glucose.

To investigate whether enhanced secretory function is intrinsic to β-cell, we assessed insulin secretion in response to glucose (2.8-16.8 mM) and KCl (20 mM) in isolated islets. Consistent with our observations of intact insulin secretion in mice with null deletion of Ceacam1 gene (DeAngelis, Heinrich et al. 2015).
glucose and KCl triggers a normal release of insulin in islets derived from $Cc1^{-/-}$ mice (Fig. 5C, upper panel). Insulin secretion in islets derived from $Cc2^{-/-}$ mice in response to KCl and physiological level of glucose (5.6 mM) is also normal (Fig. 5C, lower panel). However, $Cc2^{-/-}$ islets exhibit a minor but statistically significant reduction in insulin release in response to higher doses of glucose (11.2-16.8 mM).

Taken together, the data provide circumstantial evidence that enhanced insulin secretion in $Cc2^{-/-}$ mice is more likely secondary to a metabolic effect brought about by $Ceacam2$ deletion rather than a primary modulation of $\beta$-cell function.

Normal serum corticosterone level (Table) rules out a role for the loss of $Ceacam2$ in the anterior pituitary in the regulation of insulin secretion by the hypothalamic-pituitary-adrenal axis.

*Male $Cc2^{-/-}$ Mice are Insulin-Sensitive*

To assess insulin action, a 2-hour hyperinsulinemic-euglycemic clamp was performed. Basal glucose and hepatic glucose production at the beginning of the clamp period are similar to wild-type (Figs. 7A and 7B, respectively). Glucose infusion rate required to maintain euglycemia is normal in male $Cc2^{-/-}$ mice (Fig. 7D), indicating overall insulin sensitivity in the animal. Upon glucose infusion, insulin inhibits hepatic glucose production to the same extent in both mouse
groups (Fig. 7C). This is supported by intact phosphorylation (and activation) of IRβ and IRS2 by insulin stimulation in tissue lysates (Fig. 8D). Whole-body glycolysis and glycogen synthesis (Fig. 7E and 7F) occur at similar rates in $Cc2^{-/-}$ and $Cc2^{+/+}$ mice, which demonstrates that loss of Ceacam2 does not modify these insulin-mediated actions.

Although whole-body glucose uptake is unaltered in $Cc2^{-/-}$ mice (Fig. 7G), glucose uptake in white adipose tissue is significantly increased in male $Cc2^{-/-}$ compared to $Cc2^{+/+}$ mice (Fig. 7H). The increase in the face of a normal whole-body glucose uptake implicates reduction of glucose uptake in another tissue, most likely skeletal muscle. However, glucose uptake in gastrocnemius muscle of $Cc2^{-/-}$ mice does not reach a statistically significant reduction (Fig. 7I). Unfortunately, measuring glucose uptake in the oxidative soleus muscle is challenging, limiting the sensitivity of this assay in skeletal muscle.

Nonetheless, total insulin receptor number is decreased in gastrocnemius muscle of $Cc2^{-/-}$ mice (Fig. 8A), and subsequently insulin’s activation of its receptor was diminished, as shown by reduced insulin-stimulated receptor phosphorylation in tissue lysates (Fig. 8C). Basal phosphorylation and hence, activation of Akt is also decreased in $Cc2^{-/-}$ muscle (Fig. 8B). Lower pAKT suggests decreased glucose transport into skeletal muscle. These data indicate that insulin signaling underlying glucose transport in skeletal muscle is defective in male $Cc2^{-/-}$ mice.
**Altered Fat Metabolism in Skeletal Muscle of Cc2⁻/⁻ Mice**

In contrast with glucose uptake, fatty acid transport and oxidation appear to be elevated in skeletal muscle of Cc2⁻/⁻ mice. Fatp1 expression is elevated in Cc2⁻/⁻ mice, both at the mRNA and protein levels (Figs. 9A and 8B, respectively). In line with increased fatty acid transport, Cd36 is also increased in Cc2⁻/⁻ mice (Fig. 9A). *Ppara*, the master regulator of fatty acid oxidation genes, and one of its targets, *Cpt1*, display higher mRNA levels (Fig. 9A), suggesting enhancement of fatty acid oxidation in muscle of Cc2⁻/⁻ mice. Consistently, Cc2⁻/⁻ mice exhibit lower muscle triglyceride content (Fig. 9B). These data implicate a substrate switch in muscle of Cc2⁻/⁻ mice, favoring lipid over glucose as an energy metabolite.

In contrast to muscle, liver triglyceride content is similar to wild-type (Fig 9B), consistent with normal lipid homeostasis in Cc2⁻/⁻ livers. Together with reduced serum triglycerides content, this suggests increased uptake of triglyceride in skeletal muscle. Normal visceral adiposity and adipocyte size (not shown) and low fasting serum non-esterified fatty acids (Table), supports the preferential distribution of triglyceride to skeletal muscle, but not to adipose tissue of Cc2⁻/⁻ mice. This notion is supported by marked reduction of serum triglyceride and free fatty acids at the fed state (Fig. 9C).
Male Cc2+/ Mice Display Hyperphagia and Hyperinsulinemia in the Fed State

Loss of Ceacam2 appears to alter skeletal muscle to favor lipid over glucose metabolism. With Ceacam2 being localized to VMH (Heinrich, Ghosh et al.), a regulatory area of energy balance, we then tested whether Cc2/- mice display altered feeding behavior. Daily food intake over a period of 10 days is markedly increased in 3-4 month-old Cc2/- mice (Fig. 10A).

Consistent with enhanced $\beta$-cell function, insulin secretion (as estimated by C-peptide levels) and serum insulin level are elevated in randomly fed Cc2/- mice (Fig. 10B).

Comparable Effect of High-fat Diet on Insulin Homeostasis and Action in Male Cc2+/ and Cc2+/+ Mice

To investigate the effect of high-fat diet on the metabolic profile, male Cc2+/+ and Cc2/- mice were placed on high-fat diet (HFD) for up to 5 months. HFD causes glucose intolerance in both mouse groups (Fig. 11A). Males on HFD exhibit increases in fasting serum insulin and C-peptide levels, with a decrease in C-peptide/insulin molar ratio (Fig. 11B-D). This decrease in C/I ratio indicates decreased insulin clearance in the mice fed HFD, though the reduction is the same between the Cc2+/+ and Cc2/- mice. Insulin secretion in response to 2.0g/kg intraperitoneal glucose injection produces a normal acute phase between male Cc2+/+ and Cc2/- mice, both chow- and HFD-fed (Fig. 11E). Increased insulin
levels 2 hours after bolus on HFD demonstrates pancreatic compensation for
diet-induced insulin resistance (Fig. 11F). Male Cc2−/− mice appear to have no
greater predisposition toward insulin resistance brought about by HFD than
male Cc2+/+ mice.
DISCUSSION

CEACAM2 is a related protein to CEACAM1 (Han, Phan et al. 2001). The function of CEACAM1 in regulating insulin action by promoting insulin clearance in liver has been well documented (Rees-Jones and Taylor 1985; Poy, Yang et al. 2002; DeAngelis, Heinrich et al. 2008). The function of CEACAM2, however, remains elusive. Consistent with the differential tissue expression of Ceacam2 (Han, Phan et al. 2001; Zebhauser, Kammerer et al. 2005) in the ventromedial hypothalamus (VMH) (Heinrich, Ghosh et al.), the current studies propose an important role for this protein in the central regulation of energy balance. Mice with global deletion of Ceacam2 exhibit insulin sensitivity in the face of hyperphagia. Retained insulin sensitivity appears to be due to increased energy expenditure.

Hyperglycemic clamp analysis shows that Ce2-/- mice exhibit enhanced β-cell function, and elevation in insulin secretion in spite of decreased β-cell mass. The mechanism of reduced β-cell mass is unclear. However, given that CEACAM2 is upregulated in regenerating islets (personal communication with N. Sarvetnick at Scripps, San Diego), it is possible that Ceacam2 deletion reduces islet cell regeneration, along with its mass. This hypothesis requires further investigation. Nevertheless, it is intriguing that Ceacam2 deletion enhances insulin secretion from pancreatic β-cell. The mechanism of elevated insulin
secretion is not yet clear. Normal glucagon and somatostatin levels exclude a role for insulin antagonists in this process. Similarly, plasma corticosterone level is intact, ruling out a potential defect in the regulation of insulin secretion by the hypothalamic-pituitary-adrenal axis. Data shows that insulin secretion is not intrinsically modified in Cc2−/− islets, therefore it is more likely that enhanced insulin secretion in Cc2−/− mice is secondary to a metabolic effect brought about by Ceacam2 deletion, rather than by primary modulation of β-cell function.

Insulin secretion is regulated by sympathetic activity of signals from VMH (Landsberg and Krieger 1989), a main glucose-sensing site in the brain (Silver and Erecinska 1998; Cotero and Routh 2009). Localization of Ceacam2 to VMH and its upregulated expression at fasting (Heinrich, Ghosh et al.), a state characterized by low insulin release, provide circumstantial evidence that increased insulin secretion in Cc2−/− mice is caused by Ceacam2 deletion in VMH. Because VMH is implicated in the regulation of food intake, hyperphagia in Cc2−/− mice points to an important role of CEACAM2 in feeding behavior. It is possible that deletion of Ceacam2 from VMH leads to hyperphagia, which in turn leads to increase in insulin release, as manifested by the increased level of insulin at fed, but not fasting, state. Consistent with the positive effect of insulin on the expression of fatty acid synthase (Wilson, Back et al. 1986), hypothalamic mRNA level of Fasn was elevated in randomly fed, but not fasted, animals. Thus it is
also possible that elevation in hypothalamic FAS level could lead to hyperphagia, as previously shown (Chakravarthy, Zhu et al. 2007).

Nevertheless, fasting plasma insulin concentration remains normal, despite increase in insulin secretion. This is most probably related to compensatory insulin clearance, likely resulting from intact CEACAM1 expression level in the liver of Cc2+/− mice. In contrast to steady-state fasting, the mice exhibit higher insulin concentration at randomly fed state.

Despite hyperphagia male Cc2+/− mice gained body mass at a rate similar to wild-type over a lifetime (Heinrich, Ghosh et al.). Cc2+/− mice did not develop visceral obesity, and total fat mass is significantly reduced. Reduction in total fat mass was accompanied by reduction in serum and skeletal muscle and heart triglyceride content. An increase in skeletal muscle Fatp1 mRNA points to an increase in fatty acid redistribution to muscle for oxidation. Elevation in Ppara mRNA level and of its activation, as suggested by elevation in mRNA of its target genes (Cd36 and Cpt1), suggests enhanced fatty acid oxidation in the skeletal muscle of Cc2+/− mice. Given that skeletal muscle is a major site for energy expenditure, the indication of increased fatty acid oxidation together with decreased triglyceride content, suggests increased overall energy expenditure.

Consistent with other reports [Reviewed in (Randle 1998)], this enhancement of fatty acid as substrate for utilization was associated with reduced glucose uptake. Reduction in insulin level, receptor number and
receptor signaling is demonstrated by insulin receptor and its downstream effector AKT. In spite of reduced basal activation of AKT, glucose uptake in muscle during hyperinsulinemic clamp tended to be reduced without reaching statistical significance. Measurement of glucose uptake in soleus muscle would have demonstrated a negative effect on glucose uptake in muscle ex vivo. Regardless, clamp analysis revealed marked elevation in glucose uptake in white adipose tissue of Cc2/− mice. This enhanced glucose disposal and insulin sensitivity in WAT could compensate for muscle insulin resistance to maintain normal overall insulin action. Lower serum triglyceride in randomly fed state and absence of visceral obesity point to glucose utilization as substrate in WAT. This redistribution of glucose substrate from skeletal muscle to adipose tissue has been reported in mice with muscle-specific null mutation of insulin receptor or GLUT4 (Kim, Michael et al. 2000; Kim, Zisman et al. 2001; Cariou, Postic et al. 2004). It is intriguing that Cc2/− mice exhibit a marked decrease in insulin receptor, an event that could at least in part be due to insulin-mediated downregulation of its receptor (Vigneri, Pliam et al. 1978; Ronnett, Knutson et al. 1982; Standaert and Pollet 1984). The mechanism is not known.

Because Ceacam2 is not expressed in muscle or white adipose tissue, the observed phenotype must be secondary rather than being due to a primary metabolic defect in muscle (Najjar 2002). Localization of CEACAM2 to ventromedial hypothalamus (Heinrich, Ghosh et al.), together with glucose
redistribution for preferential glucose utilization by WAT and enhanced fatty acid utilization by skeletal muscle and heart, provide circumstantial evidence that Ceacam2 plays a significant role in the central nervous system control of energy balance and substrate utilization in the periphery. Activation of sympathetic nervous system signals regulate glucose uptake in brown adipose, heart and skeletal muscle, but not white adipose tissue.

The role of CEACAM2 in VMH is further supported by sexual dimorphism in the phenotype caused by global deletion. Like males, female Cc2−/− mice display hyperphagia with enhanced insulin secretion. However, unlike male mice, female Cc2−/− mice develop insulin resistance, resulting from impaired insulin action in skeletal muscle, leptin resistance and increase in total body and fat mass, and lower energy expenditure as measured by VO2 during a normal light-dark cycle (Heinrich, Ghosh et al.). In light of Ceacam2 being highly expressed in VMH, a site for dimorphic expression of estrogen receptor (ER) alpha (Scott, Tilbrook et al. 2000), which exerts an important role in regulating energy homeostasis as well as insulin sensitivity and secretion, and adipocyte growth (Ropero, Alonso-Magdalena et al. 2008), the sexual dimorphic phenotype of Cc2−/− mice suggests a potential association between Ceacam2 and ER signaling pathways. This remains to be determined.

Using global Ceacam2 knockout mouse the current study proposes a role for hypothalamic CEACAM2 in the regulation of insulin secretion and peripheral
action and in energy balance. Further studies involving tissue-specific targeting of Ceacam2 are required to elucidate the differential role of Ceacam2 in these metabolic processes.
SUMMARY

*Ceacam2* is a gene related to *Ceacam1*. The protein products of these genes exhibit a differential tissue-specific distribution. *CEACAM1* is ubiquitously expressed, and several functions have been ascribed to *CEACAM1*. Consistent with its high expression in the hepatocyte, *CEACAM1* regulates insulin action by promoting insulin clearance in liver, the major site of insulin endocytosis and degradation. In contrast to *CEACAM1*, *CEACAM2* distribution is limited to kidney, ventromedial hypothalamus, anterior pituitary, spleen and prostate. Its function has not been as well elucidated. To investigate the function(s) of *CEACAM2 in vivo*, we generated a *Ceacam2* null mouse. Characterization of the metabolic phenotype of the mice led to the following conclusions:

Using a mouse with a global deletion of *Ceacam2* gene, we herein show that *CEACAM2* plays an important role in the central regulation of energy balance and peripheral insulin action and secretion.

Male and female mice with null mutation of *Ceacam2* exhibit hyperphagia and increased insulin secretion.

Consistent with dimorphic regulation of metabolic and energy parameters by VMH, *Ceacam2* deletion causes a sexual dimorphic metabolic phenotype: Male mice are insulin sensitive resulting from increased energy expenditure, and
female mice develop insulin resistance due to reduced energy expenditure in muscle.
FIGURE LEGENDS

Figure 1

Generation and genotyping of Cc2+/+ and Cc2−/− mice. (A) Targeting vector for the Cc2−/− mice. (B) Southern blot of digested genomic DNA produces the wild type Cc2 allele at 6.7 kb, while the Cc2 knockout allele is 8.2 kb in size. The PCR analysis was designed with primers in the 5’ promoter region and exon 2 (named S-107 and α-123, respectively) to produce a 950 bp fragment from the Cc2 wild type allele. (C). Primers in the Neor cassette and exon 2 of the Cc2 knockout allele (S-Neor and α-123, respectively) produce a ~1.9 kb fragment.

Figure 2

Confirmation of Ceacam2 knockout. (A) PCR and Southern blot on liver from wild-type and Cc2−/− mice (n=3). (B) Protein levels of CEACAM1 are normal in liver of Cc2−/− mice (n=3). (C) Southern blot of wildtype and Cc2−/− mice with probe specific to Ceacam2. Representative figure shown. (D) Protein levels of CEACAM1 are normal in kidney of Cc2−/− mice. Representative figure of 3 mice shown. (E) Reverse transcription and PCR of isolated pancreatic islets from wild-type, Cc1−/− mice and Cc2−/− mice.
Figure 3

Lean mass and fat mass in male Cc2+/− mice measured by 1H-MRS (n=9).

All values shown ±SE. *p < 0.05.

Figure 4

Immunohistochemistry of CEACAM2 in the pancreas. Colocalization of CEACAM2 (green) with insulin, glucagon, and somatostatin (red) in wildtype and Cc2+/− mice. Colocalization is shown in yellow.

Figure 5

(A) Serum insulin levels of 6-month old male Cc2+/+ and Cc2−/− mice in response to secretogouges. (i) Mice were injected IP with 2.0 g/kg glucose and blood was removed via retroorbital sinus following the injection (n=9). (ii) Mice were challenged with 0.5g/kg L-arginine (n=7). All values shown ± SE. *p<0.05.

(B) Beta-cell area of 3-month males fasted overnight calculated by morphometric analysis. (C) Insulin secretion in isolated islets from wild-type, Cc1+/− and Cc2+/− male mice in response to various concentrations of glucose (2.8, 5.6, 11.2 or 16.8mM) or to 20mM KCl.
Figure 6

Hyperglycemic clamp. (A) Plasma glucose levels during the two hour hyperglycemic clamp (n=7). (B) Glucose infusion rate during clamp (n=7). (C) Plasma insulin levels during clamp (n=7). All values shown ±SE. *p<0.05.

Figure 7

Hyperinsulinemic-euglycemic clamp. Basal glucose (A) and hepatic glucose production (B) at the start of the clamp (n=9 and n=7, respectively). Percent inhibition of hepatic glucose production after infusion of insulin (C, n=7). Glucose infusion rate (D, n=7), glycolysis (E, n=7), and glycogen synthesis (F, n=7) during the clamp. Whole-body glucose uptake (G, n=7), glucose uptake into the white adipose tissue (H, n=8) and gastrocnemius skeletal muscle (I, n=8) during the clamp. All values shown ±SE. *p<0.05.

Figure 8

Insulin resistance in the muscle of Cc2-/ mice. (A) Fasting levels of Irβ normalized to Actin. (B) Fasting levels of FATP-1, pAKT normalized to Actin and AKT respectively (n=3). (C) Insulin stimulated insulin receptor phosphorylation in muscle of wildtype and Cc2-/ mice normalized to total insulin receptor levels (n=3). (D) Insulin-stimulated insulin receptor
phosphorylation in liver of wildtype, $Cc1^{-/-}$ mice and $Cc2^{-/-}$ mice normalized to total insulin receptor levels (n=2).

**Figure 9**

Altered lipid metabolism in muscle of $Cc2^{-/-}$ mice. (A) Real-time PCR data on fasted 6 month old gastrocnemius normalized to 18S (n=6). (B) Triglyceride content in gastrocnemius (i) muscle and liver (ii) at the end of hyperinsulinemic-euglycemic clamp (n=6). (C) Randomly fed serum triglycerides (i, n=5) and free fatty acids (ii, n=5).

**Figure 10**

(A) For food intake mice were housed individually, and food mass was weighed daily at 1200 (n=8). (B) Randomly fed serum insulin and C-peptide (n=5).

**Figure 11**

Male $Cc2^{-/-}$ mice on high fat diet. (A) IPGTT using 2.0 g/kg glucose bolus (n=8). Fasting serum insulin (B), C-peptide (C), and molar C-peptide to insulin ratio (D) (B-D n=7). Acute (E) and secondary phase (F) serum insulin following 2.0 g/kg glucose bolus (n=6). All values shown ±SE. *p<0.05.
**TABLE.**

<table>
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<tr>
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<th>Wild-type</th>
<th>$Cc2^{-/-}$</th>
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<tr>
<td>Insulin (pM)</td>
<td>57.3 ± 4.19</td>
<td>63.0 ± 5.77</td>
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<td>C-peptide (pM)</td>
<td>349 ± 38.3</td>
<td>670. ± 91.5 *</td>
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<td>C/I ratio (AU)</td>
<td>5.98 ± 0.442</td>
<td>10.3 ± 0.962 *</td>
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<td>Fasting Glucose (mg/dL)</td>
<td>106 ± 3.49</td>
<td>116 ± 4.65</td>
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<td>Glucagon (pg/mL)</td>
<td>98.3 ± 2.40</td>
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<td>Somatostatin (pg/mL)</td>
<td>819 ± 9.86</td>
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<td>Corticosterone (ng/mL)</td>
<td>4.98 ± 0.151</td>
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<td>Leptin (ng/mL)</td>
<td>3.11 ± 0.359</td>
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<td>Triglycerides (mg/dL)</td>
<td>75.8 ± 6.20</td>
<td>42.1 ± 4.92 *</td>
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<td>Free fatty acids (mEq/L)</td>
<td>0.722 ± 0.0405</td>
<td>0.497 ± 0.0687 *</td>
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N≥10 for all parameters

*P<0.05 compared to WT
Figure 1.

A. Gene Construct

i.  

```
XE X X S 1 2 ESE S N 3 4 5 6 7 8 9
```

6.7 Kb

ii.  

```
XE X S 2
```

Neo^r

iii.  

```
XE X S 2
```

Neo^r

```
EcoR1(Int 2)
```

8.2 Kb

Southern blot probes

PCR Primers

KO: S-Neo^r

WT: S-107

α-123

B. Southern Blot

Cc2 Genotype

Cc2 KO Allele

8.2 kb

Cc2 WT Allele

6.7 kb

1.9 kb

Cc1 WT Allele

950bp

Mice

150bp

C. PCR

Cc2 Genotype

Cc2 KO Allele

1.9 kb

Cc2 WT Allele

950bp

Cc1 WT Allele

150bp

Primer Specificity

CC1 WT

CC2 WT

CC2 KO
Figure 2.

A. Liver: Southern Blot Analysis

i. Primers:

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<tr>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>-/-</td>
<td>-/</td>
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EB staining

ii. Southern Blot

B. Islets: RT-PCR


C. Western Blot

i. Kidney

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ii. Liver

Mouse

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<th>Cc2&lt;sup&gt;−/−&lt;/sup&gt;</th>
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</tbody>
</table>

lb: α-CC1

ip: α-CC1
Figure 3.

* $P<0.05$ compared to WT
Figure 4. IHC using mCEACAM2 Antibody

CC2 WT        CC2⁻/-

Insulin

Glucagon

Somatostatin
Figure 5.
A. Insulin Secretion
   i. In Response to Glucose
   ii. In Response to Arginine

B. Beta-cell Area
in Male 3 month-old Cc2 mice

C. Insulin Secretion
in Isolated Islets
   i.
   ii.

* P < 0.05 compared to WT
Figure 6.

A

Plasma Glucose (mg/dL)

WT

Cc2<sup>−/−</sup>

Time (min)

B

Glucose Infusion Rate (mg/kg/min)

WT

Cc2<sup>−/−</sup>

Time (min)

C

Plasma Insulin (mU/mL)

WT

Cc2<sup>−/−</sup>

Time (min)
Figure 7.

A  
Basal Glucose (mg/dL)  

test

B  
Basal HGP (mg/kg/min)  

test

C  
% Inhibition Hepatic Glucose Production  

test

D  
Glucose Infusion Rate (mg/kg/min)  

test

E  
Glycolysis (mg/kg/min)  

test

F  
Glycogen Synthesis (mg/kg/min)  

test

G  
Glucose Uptake (mg/kg/min)  

test

H  
WAT Glucose Uptake (nmol/g/min)  

test

I  
Gastrocnemius Glucose Uptake (nmol/g/min)  

test

*  
P<0.05 compared to WT
Figure 8.

A. Muscle

WT  Cc2 −/−

Mouse

IB: α-FATP1
IB: α-pAKT
IB: α-AKT
reIB: α-ACTIN

B. Muscle

WT  Cc2 −/−

Mouse

IB: α-FATP1
IB: α-pAKT
IB: α-AKT
reIB: α-ACTIN

C. Muscle

Insulin Mouse

WT  Cc2 −/−

1 2 3

i. IP: α-IRβ  IB: α-pTYR

ii. IB: (I) with α-IRβ

D. Liver

Cc1 +/+  Cc1 −/−  Cc2 +/+  Cc2 −/−

Insulin Mouse

1 2 1 2

i. IP: α-IRβ  IB: α-pTYR

ii. IB: (I) with α-IRβ

iii. IP: α-IRS2  IB: α-pTYR

48
Figure 9.
A. Real-time PCR in Gastrocnemius

- Gene/GAPDH (Fold Induction)
  - FATP1
  - CD36
  - PPARα
  - CPT1

B. Tissue Triglyceride content

- i. Muscle
- ii. Liver

C. Serum Lipids

- i. Triglyceride
- ii. Free Fatty Acid

* P<0.05 compared to WT
Figure 10.

A. Daily Food Intake

B. Serum Hormones

* $P<0.05$ compared to WT
Figure 11.

A

Whole Blood Glucose (mg/dL) vs. Post Glucose (minutes)

- WT RD
- WT HF
- Cc2−/− RD
- Cc2−/− HF

B

Fasting Insulin (pM x 100)

- WT RD
- WT HF
- Cc2−/− RD
- Cc2−/− HF

C

Fasting C-Peptide (pM x 100)

- WT RD
- WT HF
- Cc2−/− RD
- Cc2−/− HF

D

C-Peptide / Insulin Molar Ratio

- WT RD
- WT HF
- Cc2−/− RD
- Cc2−/− HF

* P<0.05 compared to RD, † P<0.05, compared to WT

E

Serum Insulin (ng/mL)

- WT RD
- WT HF
- Cc2−/− RD
- Cc2−/− HF

F

Serum Insulin (ng/mL)

- WT RD
- WT HF
- Cc2−/− RD
- Cc2−/− HF

Post Glucose (minutes)
**MATERIALS AND METHODS**

*Generation of Targeting Vector and Ceacam2+/− Mouse*

To generate the targeting vector we subcloned a 7.338 kb *Eco*RI fragment of the mouse genomic DNA from the original BAC clone into the pGEM9Z vector [pGEM9Z(72-*Eco*RI)]. The *Eco*RI fragment contains 3.735 kb of the proximal *Ceacam2* (*Cc2*) promoter, exon 1 (64bp), exon 2 (360 bp) and the intervening intronic sequences [intron 1 (766 bp) and ~2.3 kb of intron 2]. We subcloned this fragment to the *Sac*I site 3’ to the Neor-derived sequence. This fragment represents the right arm of the targeting vector. Next, we isolated a 3.6 kb *Xba*I fragment containing the mouse *Cc2* gene promoter and cloned it into the *Xba*I site of plasmid pGK Neor 5’ to the Neor cassette. The 3.6 kb *Xba*I fragment represents the left arm of the targeting vector. We linearized the plasmid with *Apa*I prior to electroporation into J-1 ES cells. We identified two positive clones out of 200 by Southern blotting, and we injected ES cells into blastocysts. Two resultant chimera mice were bred with C57BL/6 mice and *Cc2*+/− pups were identified by PCR. *Cc2*+/− mice were backcrossed twice with C57BL/6 mice resulting in a mixed C57BL/6x129sv background. All animals were housed in a 12 hour dark-light cycle and fed either standard chow or high fat diet (Research
Diets #D12451) ad libitum. All procedures were approved by the Institutional Animal Care and Utilization Committee.

**Genotyping by Southern Blot and Polymerase Chain Reaction**

For genotyping by Southern blot (Fig. 1B), genomic DNA from mouse tail was restriction digested XbaI and NsiI and separated by gel electrophoresis followed by transfer to nitrocellulose membrane. Radiolabeled 32P-dCTP was incorporated into cDNA probes for the neomycin cassette or intron 2 and hybridized to the membrane. Hybridization was visualized by autoradiogram. For genotyping by PCR (Fig. 1C), mouse tail was digested at 55°C for 4 hours in DirectPCR (Tail) lysis buffer (Viagen) and 0.4g/L Proteinase K (Roche). Tail lysate DNA was amplified using HotStarTaq polymerase (Invitrogen) under the following conditions: 1 cycle (95°C x 15 minutes), 36 cycles (94°C x 30 seconds, 62°C 30 seconds, 72°C 3 minutes), 1 cycle 72°C x 10 minutes.

Primers used to amplify the *Ceacam2* wild-type allele are:
Forward (-107) 5'- GCC CTT CTC TGG GAG GAG AAT CAA T -3'
Reverse (123) 5'- GCC TTC AGC AGC GTG GAG TGG A -3"'

Primers used to amplify the *Ceacam2* knockout allele are:
Forward (SNeo9) 5'- GGA TCG GCC ATT GAA GAA GAT -3'
Reverse (123) 5'- GCC TTC AGC AGC GTG GAG TGG A -3”.

The *Ceacam1* wild type allele was amplified using primers:
Forward (S-PCC1) 5'- CCT TGC TGC TGG AGT ATG TT -3’
Islet Isolation, Reverse Transcription, and Northern Blot for Ceacam2

Pancreatic islets were purified by collagenase digestion as previously described (DeAngelis, Heinrich et al., 2008). Liver, kidney, and islet messenger RNA (mRNA) were isolated using TRIzol (Invitrogen) followed by the MicroPoly (A) Pure kit (Ambion) per manufacturer instructions. Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen) followed by PCR using \textit{Ceacam1} (116 and 179) and \textit{Ceacam2} (117 and 180) specific primers as previously described (Han et al., 2001). PCR products were separated by gel electrophoresis, stained with ethidium bromide for visualization, and transferred to nitrocellulose membrane. 32P-labeled oligonucleotide probes 181 and 182 (Han et al., 2001), specific for \textit{Ceacam1} and \textit{Ceacam2} respectively, were hybridized to the membrane and visualized by autoradiogram as previously described (Han et al., 2001). Kidney mRNA was separated by formaldehyde gel electrophoresis and transferred to a nitrocellulose membrane followed by hybridization to Random Primed (Roche) complimentary DNA (cDNA) specific for the exon2 region of \textit{Ceacam2}, followed by hybridization to a β-actin cDNA probe for normalization (DeAngelis, Heinrich et al., 2008).

Immunoprecipitation and Western Blot for Ceacam1
Liver and kidney tissue lysates from Ceacam2+/+, +/-, or -/- mice were immunoprecipitated with polyclonal antibody specific to CEACAM1 (α-mCC1, Ab 231) (Najjar et al., 2005), followed by binding the complex to protein A agarose (Invitrogen), separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transfer to a nitrocellulose membrane (Whatman). The membrane was then probed with the mouse specific CEACAM1 antibody (Ab-231) and anti-rabbit horseradish-peroxidase-conjugated anti-immunoglobulin G antibody (Amersham), and visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Body Composition

Total fat mass and lean mass were measured by ¹H-magnetic resonance spectroscopy (MRS, Bruker Mini-Spec Analyzer; Echo Medical Systems) as previously described (Park et al., 2006).

Measurements of Serum Metabolites

Serum insulin, C-peptide, glucagon, somatostatin, corticosterone and leptin levels were measured by radioimmunoassays (Phoenix Biotech for somatostatin, MP Biomedicals for corticosterone, Millipore for all others). Triglycerides and free fatty acids were measured by triglyceride kit (Pointe Scientific) and NEFA C kit (Wako), respectively.
**Immunohistochemistry**

Female wildtype and Cc2-/mice were fasted for 6 hours and anesthetized with pentobarbital (55 mg/kg). PBS followed by 4% paraformaldehyde was perfused through the left ventricle followed by organ removal. Pancreas tissue was paraffin embedded and sectioned. IHC was performed using affinity purified antibody specific to mCC2 peptide sequence CNAEIVRFVTGNKTIGPVH (Bethyl), followed by incubation with Antirabbit AlexaFluor 488 (green) antibody (Invitrogen). The same sections were then incubated with antibodies to insulin (Dako), glucagon (Sigma), or somatostatin (SantaCruz) followed by the appropriate AlexaFluor 555 (red) antibody. Visualization was performed using a Nikon microscope and software.

**Insulin Secretion in Response to Glucose and L-arginine**

Overnight fasted mice were anesthetized with pentobarbital (55 mg/kg) and blood was removed from the retroorbital sinus for fasting insulin measurement. A bolus of glucose (2.0 g/kg) or L-arginine (0.5 g/kg) was injected IP and blood was removed from the retroorbital sinus at 2, 7, 30, and 120 minutes after the bolus for insulin and 2, 5, 15 and 30 minutes for arginine.
**Beta-cell Area**

Mice were anesthetized, and pancreata were dissected, weighed, fixed in Bouin’s solution, sectioned, and stained. β-cell area was calculated by morphometric analysis using Image J software (National Institutes of Health; http://rsb.info.nih.gov/ij/), and the insulin-stained area was divided by total pancreas area.

**Insulin Secretion from Isolated Islets**

Islets were isolated from 6-month-old WT, Cc1−/−, and Cc2−/− mice by collagenase digestion followed by centrifugation over a Histopaque gradient. Briefly, after clamping the common bile duct at its entrance to the duodenum, 3 ml of M199 medium containing 1 mg/ml of collagenase P (Roche Molecular Biochemicals, Indianapolis, IN.) was injected into the duct. The swollen pancreas was surgically removed and incubated at 37°C for 17 min. Thereafter, 30 ml of ice-cold M199 medium containing 10% newborn calf serum (NCS) were added to stop the digestion reaction. Digested pancreata were dispersed by pipetting and rinsed twice with 30 ml of the same medium. After filtering the tissue suspension through a Spectra-mesh (400µm; Spectrum Laboratories, Inc.),
the digested tissue was resuspended in 10 ml of Histopaque and overlaid with 10 ml of M199 medium. The samples were then centrifuged at 1,700 g for 20 min, and the islets were collected from the interface. The recovered material was washed twice with cold M199 medium, resuspended in RPMI medium containing 10% NCS and 5.5 mM glucose, and cultured overnight at 37°C in 5% CO2. For insulin secretion assays, islets were manually picked under a dissection microscope using a pipette, placed in ice-cold Krebs buffer (119 mM NaCl, 2.5 mM CaCl2, 1.19 mM KH2PO4, 1.19 mM Mg2SO4, 10 mM HEPES [pH 7.4], 2% bovine serum albumin, and 2.8 mM glucose) and incubated at 37°C for 15 min. At the end of the incubation period, islets were stimulated with various glucose concentrations (2.8, 5.6, 11.2, and 16.8 mM) or 20 mM KCl for 1 h at 37°C. At the end of the incubation period, the islets were collected by centrifugation and the supernatant was assayed for insulin content by radioimmunoassay. Islets in the pellets were dissolved in high salt buffer and sonicated 3 times at 80 Watts for 10 sec and DNA concentration was determined by a spectrometry. Insulin secretion from islets was normalized to DNA content.

Hyperglycemic Clamp for in vivo Beta-cell Function

Following overnight fast, a 2-hr hyperglycemic clamp will be conducted in 5-month old awake mice with a variable infusion of 20% glucose to raise and maintain plasma glucose concentration at ~300 mg/dl. Blood samples will be
collected at 10~15 min intervals for the measurement of plasma glucose and insulin concentrations. The area-under-curve of plasma insulin levels and glucose infusion rate to maintain hyperglycemia reflect pancreatic β-cell function in vivo.

Hyperinsulinemic-euglycemic Clamp for in vivo Insulin Action

Hyperinsulinemic-euglycemic clamp was performed as previously described (Park, Cho et al. 2006; DeAngelis, Heinrich et al. 2008).

Immunoprecipitation and Western Blots

Overnight fasted mice were anesthetized with pentobarbital (55 mg/kg) and organs were removed and flash frozen. For fasting protein levels, muscle tissue was homogenized and protein separated by SDS-PAGE. Membranes were probed with α-FATP1 (Santa Cruz), α-pAKT (Cell Signaling), α-AKT (Cell Signaling), and β-Actin (Sigma) antibodies, followed by incubation with HRP-conjugated secondary antibodies (Santa Cruz) and visualization by ECL.

Ex vivo insulin stimulation was performed as previously described (DeAngelis, Heinrich et al. 2008). Immunoprecipitation with α-IRS2 (Upstate) was also performed followed by immunoblotting with α-pTYR (Upstate).

Real Time PCR
Total RNA was prepared using PerfectPure RNA Tissue kit (5Prime, Gaithersburg, MD) according to the manufacturer's protocol. cDNA was synthesized with oligo dT primers and Improm II Reverse Transcriptase (Promega, Madison, WI). Real-time quantitative PCR (RT-qPCR) was performed using Fast SYBR Green PCR mix on a StepOne Plus Real Time PCR system (Applied Biosystems, Foster City, CA). Standard curves for each primer set were generated using serial 1:4 dilutions of pooled samples. The results of RT-qPCR results are reported as fold changes in mRNA levels relative to wildtype controls in arbitrary units.

**Tissue Triglyceride Content**

Intracellular triglyceride level was measured following hyperinsulinemic-euglycemic clamp by digesting tissue samples in chloroform-methanol and performing spectrophotometry using triglyceride assay kit (Pointe Scientific).

**Daily Food Intake**

All animals were housed individually in a 12 hour dark-light cycle and provided standard chow *ad libitum*. Food in cage was weighed daily at 1200. Intake is calculated as food mass from previous day minus remaining food in cage.
**Intraperitoneal Glucose Tolerance Test**

After an overnight fast (1700 until 0800 h the next day), anesthetized mice were injected intraperitoneally with glucose (2.0 g/kg body wt), and venous blood was drawn at 0, 15, 30, 60, 120 and 180 min after injection to determine blood glucose. Whole blood glucose measurements were made with a glucometer (Accu-check, Roche).

**Statistical Methods**

Data was analyzed using GraphPad Prism 4.0 software. Statistical significance was determined by two-tailed Student t-test unless otherwise indicated. Welch’s correction was used if variances were significantly different. Statistical significance was set at 5%.
REFERENCES

growth factor receptor--mediated cell proliferation." J Clin Invest 114(7):
944-52.

contact with L-type Ca2+ channels accounts for first-phase insulin

lipogenic enzymes and insulinemia during suckling and weaning on to a

Bergman, R. N. and M. Ader (2000). "Free fatty acids and pathogenesis of type 2

arginine-induced insulin release. Functional response of islets to L-
arginine and L-ornithine." Biochim Biophys Acta 1013(2): 144-51.


augmentation mechanisms, granule pools, and biphasic insulin secretion."
Diabetes 51 Suppl 1: S83-90.


Schmoll, D., K. S. Walker, et al. (2000). "Regulation of glucose-6-phosphatase gene expression by protein kinase Balpha and the forkhead transcription factor FKHR. Evidence for insulin response unit-dependent and -


