CHEMERIN REGULATES ADIPOSITY AND ENERGY HOMEOSTASIS

by

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Abstract

Obesity, characterized by an excess of adipose tissue, is an established risk factor for cardiovascular disease and type II diabetes. Different mechanisms linking obesity with these comorbidities have been postulated but remain poorly understood. Adipose tissue secretes bioactive signaling molecules, termed adipokines, which regulate various biological functions including appetite, energy balance, glucose homeostasis, and inflammation. Chemerin is a novel adipokine that regulates adipocyte differentiation and metabolism by binding to and activating the G protein-coupled receptor chemokine like receptor-1 (CMKLR1). Herein, we have shown that serum levels of the novel adipokine chemerin are significantly elevated in mouse models of obesity/diabetes. Administration of exogenous chemerin exacerbates glucose intolerance, lowers serum insulin levels, and decreases tissue glucose uptake in obese/diabetic but not normoglycemic mice. In CMKLR1-deficient mice food consumption, total body mass, and percent body fat are lower compared to wildtype controls, regardless of diet (low or high fat). CMKLR1-/- mice also exhibited decreased hepatic and white adipose tissue TNFα and IL-6 mRNA levels coincident with decreased hepatic dendritic cell infiltration, decreased adipose CD3+ T cells and increased adipose natural killer cells. CMKLR1-/- mice were also glucose intolerant compared to wildtype mice, and this was associated with decreased glucose stimulated insulin secretion as well as decreased skeletal muscle and white adipose tissue glucose uptake. Collectively, these data provide compelling evidence that chemerin/CMKLR1 signaling influences adipose tissue development, inflammation, and glucose homeostasis and may contribute to the metabolic derangements characteristic of obesity and obesity-related diseases.

List of Abbreviations and Symbols Used

α	alpha
β	beta
δ	delta
γ	gamma
λ	lambda
ζ	zeta
2-DOG	2-deoxyglucose
ACE	angiotensin converting enzyme
AdipoR	adiponectin receptor
ADP	adenosine diphosphate
AgRP	agouti-related peptide
Akt	serine/threonine protein kinase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
ASP	acylation-stimulating protein
AUC	area under the curve
aP2	adipocyte protein 2
APS	Adaptor protein containing a PH and SH2 domain
AS160	Akt substrate of 160 kDa
ATP	adenosine triphosphate
BAT	brown adipose tissue
BMI	body mass index
C3G	CRK SH3-binding guanine nucleotide-releasing protein
cAMP	cyclic AMP
CAP	Adenylate cyclase-associated proteins

CART	cocaine and amphetamine-regulated transcript
Cbl	Cas-Br-M (murine) ecotropic retroviral transforming sequence
CCRL2	chemokine (C-C motif) receptor-like 2
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT-enhancer-binding protein
CMKLR1	chemokine-like receptor 1
CMV	cytomegalovirus
CNS	central nervous system
CoA	coenzyme A
CrkII	CT10 regulator of kinase II
CRP	C reactive protein
CSF	cerebrospinal fluid
DAG	diacylglycerol
db	diabetes
DC	dendritic cell
DEXA	dual energy x-ray absorptiometry
DGAT2	diacylglycerol o-acyltransferase 2
DIO	diet-induced obesity
DMEM	Dulbecco's modified eagle medium
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum

G6P	glucose-6-phosphate
Gab1	Grb2-associated-binding protein 1
GAP	GTPase activating protein
GLUT	glucose transporter
GPCR	G protein-coupled receptor
GPR1	G protein-coupled receptor 1
Grb2	growth factor receptor-bound protein 2
GSA	glucose specific activity
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
GTT	glucose tolerance test
H&E	hematoxylin and eosin
HDL	high-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	high fat
HOMA-IR	homeostasis model assessment of insulin resistance
IL	interleukin
ip	intraperitoneal
IRE1	inositol-requiring enzyme 1
IRS	insulin receptor substrate
IST	insulin sensitivity test
JNK	c-Jun N-terminal kinase
KO	knockout
LF	low fat
LPS	lipopolysaccharide
LV	liver
Ly-6G	lymphocyte antigen 6G

MAP	mitogen activated protein
MEK	MAPK/ERK kinase
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
ND	not detected
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NPY	neuropeptide Y
N-WASP	Neural Wiskott-Aldrich syndrome protein
ob	obesity
OH	hydroxyl
P	phosphate
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCOS	polycystic ovary syndrome
PDK1	phosphoinositide-dependent kinase 1
PH	plekstrin homology
PI	phosphoinositide
PIP	phosphatidylinositol phosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
POMC	pro-opiomelanocortin
PP1	protein phosphatase 1
PPAR	peroxisome proliferator-activated receptor
QPCR	quantitative real time polymerase chain reaction

Rab	ras analog in brain
RARRES2	retinoic acid receptor responder 2
RBC	red blood cell
RBP4	retinol binding protein 4
RNA	ribonucleic acid
RT	reverse transcription
S6K1	ribosomal protein S6 kinase
SAA3	serum amyloid A3
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SGLT	sodium-glucose cotransporter
SH2	src homology 2
SH3	src homology 3
Shc	Src homology containing protein
SHP2	SH2 domain–containing protein tyrosine phosphatase–2
SIRP	signal-regulatory proteins
SK	skeletal muscle
SKM	skeletal muscle
SNAP23	synaptosomal-associated protein 23
SNP	single-nucleotide polymorphism
SOCS3	suppressor of cytokine signaling 3
SOS	son-of-sevenless
SREBP	sterol regulatory element-binding protein
SVF	stromal vascular fraction
T2DM	type II diabetes mellitus
TAG	triacylglycerol
TBST	tris-buffered saline tween 20

TIG2	tazarotene-induced gene 2
TCF	ternary complex factor
TNF	tumor necrosis factor
TZD	thiazolidinediones
UV	ultraviolet
VAMP2	Vesicle-associated membrane protein 2
VCAM-1	vascular cell adhesion molecule-1
VCO2	volume of carbon dioxide eliminated
VLDL	very-low density lipoprotein
VO2	volume of oxygen consumption
W6	week 6
W24	week 24
WA	white adipose
WAT	white adipose tissue

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Chapter I: Introduction

1.1 Obesity

Obesity, characterized by an excess of dysfunctional adipose tissue, is a rapidly growing problem that has reached epidemic proportions worldwide, particularly in highly industrialized societies of North America and Europe [1]. Obesity is commonly defined in terms of the body mass index (BMI); the division of weight in kilograms by the square of height in meters [2]. Since BMI does not take into account body composition or fat distribution of the body, it may not be an accurate predictor of health risk for certain groups, such as extreme athletes. Because of this, waist to hip ratio is also commonly used as a supplementary measure to identify additional health risk [3].

In 2005, approximately 23.2 % of the world population was overweight, and 9.8 % was obese [4]. Recent projections, adjusting for secular trends, predict that the prevalence of overweight and obese population in 2030 will increase to 38.1 % and 19.7 % respectively [4]. The prevalence of overweight and obese individuals in highly industrialized societies, such as Canada, is even greater. Between 1979 and 2004, the prevalence of overweight Canadian adults increased from 35.4 % to 36.1%, and the prevalence of obese adults increased from 13.7 % to 23.0 % [5]. In 2009, the prevalence of obesity in Canada increased to 24.1 % [6]. Obesity can be further characterized into three classes according to the increased health risks associated with increasing BMI levels: class I (BMI 30– 34.9), class II (BMI 35– 39.9) and class III (BMI $\geq$ 40). Studies of the distribution of obesity continually confirm that the BMI has been increasing over time, but more so at the higher BMI/higher risk level than at the lower BMI/lower risk levels. For example, between 1979 and 2009, class I obesity increased approximately 1.4 fold from 10.5 % to 15.1 %, class II obesity increased 2.5 fold from 2.3 % to 5.8 %, and

class III obesity, the most severe form, increased 3.4 fold from 0.9 % to 3.1 % [5, 6], much higher than the increase in overweight and class I obesity. Unfortunately, childhood and adolescent obesity rates parallel those of adults and evidence has demonstrated that once children become obese, interventions to reverse these conditions are rarely successful [7]. In one study, approximately 75 % of obese children remained obese as adults, and with a BMI greater than adults with adult-onset obesity [8]. In contrast, only 7 % of normal-weight children became obese adults [8]. Owing to the increasing rates of childhood obesity, it is predicted that the global life expectancy in North America will decline for the first time in recent history [9].

The economic burden of obesity may be grouped into direct and indirect costs. Direct costs include the cost of treatment, care and rehabilitation and indirect costs include reductions in economic productivity stemming from the poorer health, absenteeism, disability and premature mortality that are a result of obesity and obesity-related diseases. In 2006, the consequent direct cost of being overweight and obese in Canada was estimated to be $6.0 billion ($2.0 billion for overweight, and $4.0 billion for obese individuals), and indirect costs attributable to being overweight and obese were $5.0 billion ($1.8 billion for overweight and $3.2 billion for obese individuals) [10].

Obesity is associated with a variety of metabolic derangements that increase the risk for developing a number of diseases including hypertension, cardiovascular disease, and type II diabetes [11, 12]. Despite the clear link between obesity and the development of these prevalent diseases, the mechanisms responsible for this relationship are not fully understood. In addition to serving a role as an important energy storage organ, adipose tissue secretes a number of bioactive signaling molecules known as adipokines [13, 14]. Through endocrine actions in various tissues of the body, adipokines affect many

physiological processes, including glucose homeostasis [14, 15]. The synthesis and secretion of many adipokines is dynamic and modifiable, with levels of pro-inflammatory/pro-diabetic adipokines commonly increasing with obesity, and levels of anti-inflammatory/anti-diabetic adipokines decreasing with obesity [16]. In obese individuals, changes in the secretion of adipokines that are involved in insulin sensitivity have been shown to impact glucose homeostasis and the development of insulin resistance [17-19].

1.2 Glucose Homeostasis

Glucose is used by all cells of the body as a source of metabolic energy, and in biosynthetic reactions. Throughout a 24 h period, blood glucose levels are generally maintained between 4 – 8 mmol/L as blood glucose levels outside this range can result in numerous adverse effects. Hypoglycemia is a state characterized by lower than normal levels of blood glucose, and hyperglycemia is a characterized by a higher than normal level of blood glucose. Due to the dependence of the brain on glucose as an energy source, the principal symptoms of hypoglycemia arise from an inadequate supply of glucose to the brain, resulting in impaired neurological function. During hyperglycemia, most cells are able to reduce the transport of glucose into the cell, resulting in a constant internal glucose concentration. The cells most susceptible to damage by hyperglycemia are those that are unable to maintain a constant internal glucose concentration, such as endothelial cells and mesangial cells. This results in diabetic complications such as diabetic nephropathy, neuropathy, and retinopathy [20, 21]. Thus glucose homeostasis has evolved as a highly regulated process as to avoid sustained fluctuations in blood glucose levels. To ensure a constant supply of glucose to the brain and other tissues, metabolic

fuels capable of producing glucose are stored for use in times of need. Glucose is stored as glycogen in liver and muscle tissue. However, skeletal muscle cells lack the enzyme glucose-6-phosphatase, which is required to dephosphorylate glucose prior to release into the blood. Thus muscle cell glycogen functions as a reserve of glucose for skeletal muscle tissue that is not shared with other body tissues. Since the liver does express glucose-6-phosphatase, liver glycogen is the main supplier of glucose for other tissues. When cellular demand for glucose causes glycogen store depletion, the liver transforms noncarbohydrate compounds into glucose using the process of gluconeogenesis. Skeletal muscle tissue contributes to this process by metabolizing glucose-6-phosphate to pyruvate and lactate under aerobic and anaerobic conditions respectively, which are used by the liver as substrates in gluconeogenesis. Adipose tissue contributes by breaking down triacylglyceride stores into fatty acids and glycerol. Glycerol can be used by the liver as a substrate for gluconeogenesis, while fatty acids undergo β-oxidation in most tissues to drive energy production [22].

In mammals, glucose uptake is mediated by energy-coupled sodium-glucose cotransporters (SGLTs) and facilitative mechanisms by the protein family of glucose transporters (GLUTs). The active SGLT transport is responsible for the absorption and reabsorption of glucose from food within the gastrointestinal tract and urine in the kidney respectively. Glucose homeostasis within the tissues is primarily maintained by members of the GLUT protein family [23, 24]. Based on primary sequence similarities, the GLUT transporter family is divided into three subclasses with a total of 14 isoforms. The members of the class II and III subfamilies have only recently been identified, and the function of these new glucose transporter isoforms has not been clearly defined. Class I is comprised of the classical, well characterized glucose transporters GLUT1-4 and

GLUT14 [25, 26]. GLUT1, though not expressed in hepatocytes, is the most ubiquitously expressed isoform and is responsible for the basic supply of cells with glucose and transporting glucose across the blood-brain barrier [27, 28]. GLUT2 is expressed in liver, intestine, kidney, and pancreatic β-cells [29]. In the intestine and kidney, GLUT2 is involved in the absorpotion and reabsorption of glucose [30, 31]. Glucose uptake into liver tissue is principally facilitated by GLUT2 [23]. In pancreatic β-cells, GLUT2 facilitates glucose uptake into the cell, and is critical in modulating insulin secretion. As a regulator of insulin secretion, GLUT2 also plays an important role in glucose uptake of skeletal muscle and white adipose tissue. GLUT3 is a high-affinity glucose transporter, that is generally considered a neuron-specific transporter due to being highly expressed in the brain [32-35]. GLUT14 shares 95 % primary sequence similarity with GLUT3, and appears to be a consequence of gene duplication [36]. However, unlike GLUT3, GLUT14 is exclusively expressed in the testis, and has no ortholog in mice [36]. GLUT4, one of the most studied glucose transporters, translocates to the cell surface following insulin stimulation in skeletal muscle and white adipose tissue, and thus plays an essential role in insulin-stimulated glucose uptake and the development of insulin resistance and type II diabetes [37].

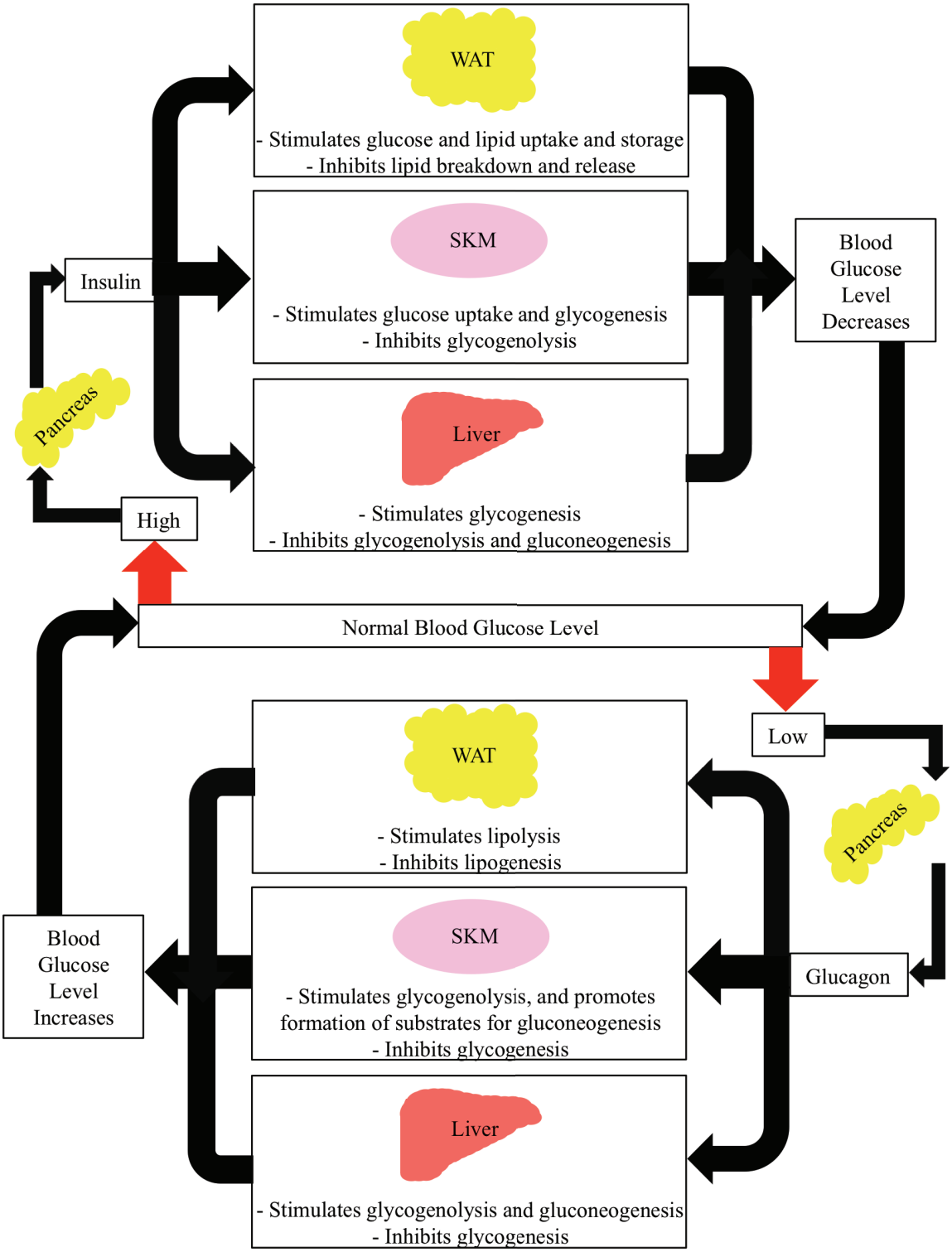
1.3 Glucose Sensing and Insulin Secretion

The adverse effects of both hypo- and hyperglycemia have resulted in a highly regulated process for monitoring and modulating blood glucose levels. Glucose homeostasis is controlled primarily by the anabolic hormone insulin. Insulin is a polypeptide hormone synthesized in β-cells of the islets of Langerhans in the pancreas.

Insulin is the most potent physiological anabolic agent known, promoting the storage and synthesis of lipids, proteins, and carbohydrates and inhibiting their breakdown and release into circulation. Catabolic hormones, such as glucagon, oppose the action of insulin. A decrease in blood glucose concentration causes an immediate secretion of glucagon from pancreatic α-cells, which induces hepatic glycogenolysis and the release of glucose into the blood (Figure 1). Evidence that α-cells directly sense and respond to a decline in blood glucose levels is contradictory [38-40], however studies have shown that nuclei in the hypothalamus and brainstem detect decreases in blood glucose concentration, and respond by stimulating glucagon secretion from pancreatic α-cells [41-45]. An increase in blood glucose concentration triggers secretion of insulin from pancreatic β-cells (Figure 1).

The primary means by which insulin increases energy storage or utilization is the regulated transport of glucose into the cell in striated (skeletal and cardiac) muscle and adipose tissues, mediated by the facilitative glucose transporter GLUT4. The appropriate secretion of insulin from β-cells is essential for proper energy homeostasis. Pancreatic β-cells have evolved to sense blood glucose levels and adjust insulin secretion as required. As blood glucose levels increase, the passive transport of glucose into β-cells by GLUT2 increases (Figure 2). Rather than activating specific receptor molecules, glucose enters the glycolysis pathway to generate downstream signals that stimulate insulin secretion. As intracellular glucose concentrations increase, the rate of glycolysis increases, ATP levels increase, and the ATP/ADP ratio increases [46, 47]. The increase in intracellular ATP/ADP causes ATP-sensitive K^+ channels to close, resulting in an increase in

Figure 1. The Opposing Actions of Insulin and Glucagon. An increase or decrease in blood glucose levels results in secretion of insulin from pancreatic beta cells, or glucagon from pancreatic alpha cells, respectively. These hormones act on tissues such as white adipose tissue (WAT), skeletal muscle (SKM), and liver, to return blood glucose levels to normal.

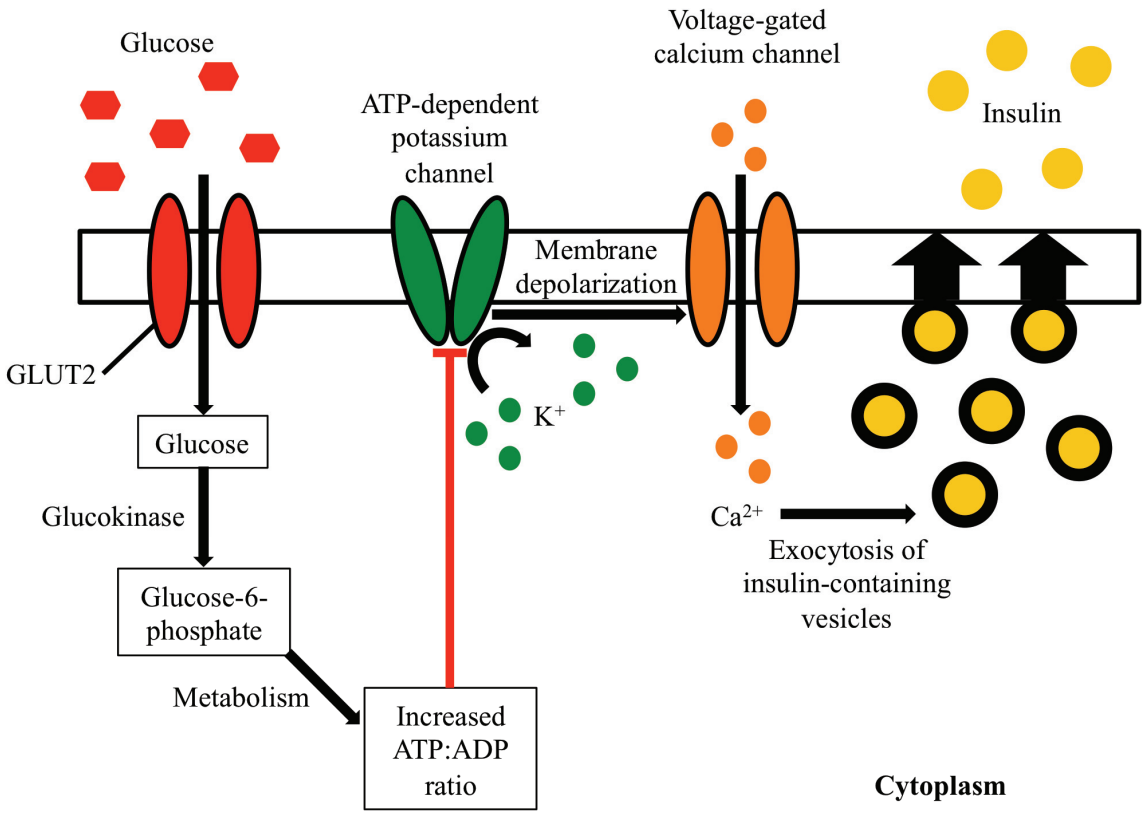

WAT
- Stimulates glucose and lipid uptake and storage
- Inhibits lipid breakdown and release
SKM
- Stimulates glucose uptake and glycogenesis
- Inhibits glycogenolysis
Liver
- Stimulates glycogenesis
- Inhibits glycogenolysis and gluconeogenesis
Insulin
Pancreas
High
Blood Glucose Level Decreases
Normal Blood Glucose Level
Low
WAT
- Stimulates lipolysis
- Inhibits lipogenesis
SKM
- Stimulates glycogenolysis, and promotes formation of substrates for gluconeogenesis
- Inhibits glycogenesis
Liver
- Stimulates glycogenolysis and gluconeogenesis
- Inhibits glycogenesis
Pancreas
Glucagon
Blood Glucose Level Increases

intracellular K^+ and depolarization of the plasma membrane. Depolarization causes an influx of extracellular Ca^{+2} through voltage-gated Ca^{+2} channels, resulting in a sharp increase in intracellular Ca^{+2} concentration. Elevated Ca^{+2} levels activate protein motors and kinases, which then mediate exocytosis of insulin-containing vesicles [47-49]. When blood glucose levels decline, glucose transport into β-cells decreases, the ATP/ADP ratio decreases, ATP-sensitive K^+ channels open. This results in cell membrane repolarization, and cessation of insulin secretion. Using a plasma membrane Ca^{+2}-ATPase transporter and a Na^+/Ca^{+2} exchanger, intracellular calcium levels are returned to basal levels [50, 51].

1.4 Insulin Signaling and GLUT4 Translocation

GLUT4 is the only isoform of the GLUT transporter family that has been established as being insulin responsive. GLUT4 is localized to intracellular compartments including the trans-Golgi-network and recycling endosomes. GLUT4 protein slowly but continually cycles between the cell surface and the intracellular compartments, with approximately 2-5 % residing at the cell surface in the basal state [52, 53]. The majority of GLUT4 remains within the cell in the absence of insulin because of the slow rate of GLUT4-containing vesicle exocytosis compared to endocytosis. Activation of the insulin receptor results in an increase in the exocytic rate and a decrease in the endocytic rate of GLUT4, essentially redistributing GLUT4 to the membrane [54-56]. Once at the membrane, GLUT4 facilitates the diffusion of circulating glucose down a concentration gradient into the cell. In the cell, glucose is rapidly phosphorylated by glucokinase to form glucose-6-phosphate, which then enters glycolysis. Once blood glucose levels return to normal range, circulating insulin returns to basal levels and GLUT4 is rapidly

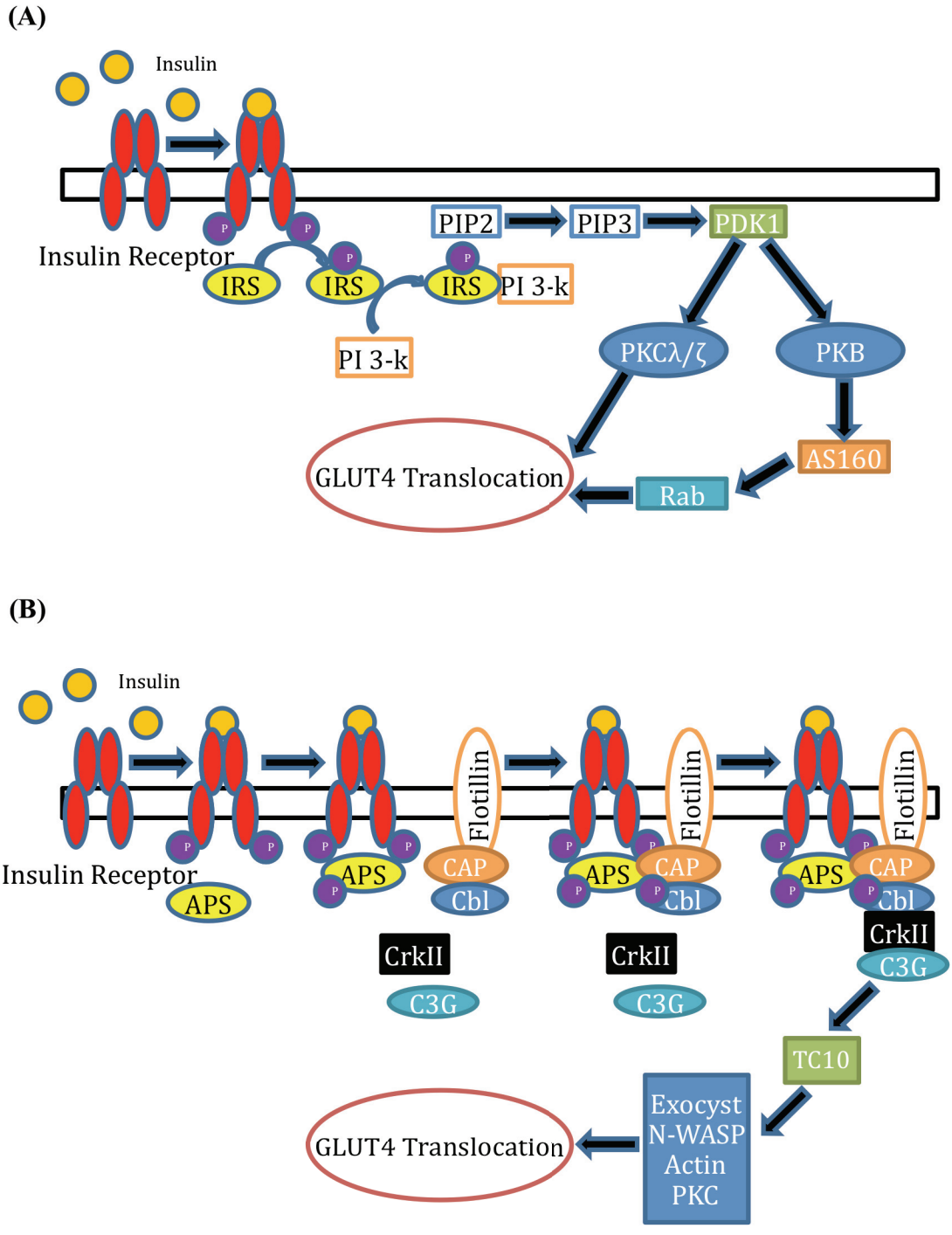
Figure 2. Insulin Secretion from Pancreatic β-Cells. GLUT2 mediates glucose uptake into β-cells. Phosphorylation of glucose to glucose-6-phosphate by glucokinase and subsequent metabolism of glucose-6-phosphate increases the ATP to ADP ratio in the cytoplasm, causing the closure of ATP dependent potassium channels. Intracellular accumulation of potassium results in membrane depolarization, causing voltage-gated calcium channels to open. The increase in intracellular calcium concentration induces exocytosis of insulin-containing vesicles.


Glucose
ATP-dependent potassium channel
Voltage-gated calcium channel
Insulin
Membrane depolarization
GLUT2
K+
Glucose
Glucokinase
Glucose-6-phosphate
Metabolism
Increased ATP:ADP ratio
Ca2+
Exocytosis of insulin-containing vesicles
Cytoplasm

internalized mainly through clathrin-dependent endocytosis and recycled back into intracellular compartments, thereby terminating facilitated glucose uptake.

When glucose enters the blood stream from the intestine following feeding, pancreatic β-cells release insulin containing vesicles by exocytosis. Insulin then directs other cells of the body to take up glucose by activating the insulin receptor. The insulin receptor is a member of a family of transmembrane receptors with intrinsic tyrosine kinase activity. Unlike other members of this family, the insulin receptor consists of two extracellular α subunits and two transmembrane β subunits linked by a disulfide bond into an α2β2 heterotetrameric complex [57]. Insulin binding to the α subunit induces a conformational change that causes transphosphorylation of one β subunit by the other on specific tyrosine residues in an activation loop, resulting in the increased catalytic activity of the kinase [58]. Rather than directly recruiting downstream effector molecules to its phospho-tyrosine residues, the insulin receptor uses scaffolding proteins which then recruit downstream effector proteins, including insulin receptor substrate (IRS) 1-4, Gab1, Shc, SIRPs, Cbl, and APS [59]. Phosphorylation of the IRS proteins by the insulin receptor generates docking sites for many downstream effector molecules containing Src homology 2 (SH2) domains that specifically recognize different phosphotyrosine motifs [60]. These proteins often contain Src homology 3 (SH3) domains that bind proline-rich regions, thus acting as binding sites for further downstream effector molecules. There are two well documented independent insulin signaling cascades that are involved in insulin-dependent GLUT4 translocation. The first, and most established pathway is the IRS-PI 3-kinase pathway (Figure 3A), and the second pathway interacts with the APS-CAP-Cbl protein complex that is located in lipid rafts/caveolae that are particularly prominent in adipocytes (Figure 3B)[61, 62].

Figure 3. Insulin Stimulates Glucose Uptake by Causing GLUT4 Translocation to the Cell Membrane. Shown here is a simplified representation of the molecular mechanisms involved in the **(A)** IRS-PI 3-kinase pathway and **(B)** the APS-CAP-Cbl protein complex that is located in lipid rafts/caveolae. P: phosphorylated species.


(A)
Insulin
Insulin Receptor
IRS
IRS
IRS
PI 3-k
PI 3-k
PIP2
PIP3
PDK1
PKCλ/ζ
PKB
AS160
Rab
GLUT4 Translocation
(B)
Insulin
Insulin Receptor
APS
APS
APS
APS
Flotillin
Flotillin
Flotillin
CAP
CAP
CAP
Cbl
Cbl
Cbl
CrkII
CrkII
CrkII
C3G
C3G
C3G
TC10
Exocyst
N-WASP
Actin
PKC
GLUT4 Translocation

PI 3-kinase is involved in numerous signaling pathways and controls key functions of the cell [63]. PI 3-kinase is the most widely accepted signaling enzyme necessary for insulin-stimulated glucose uptake. Type 1A PI 3-kinase is a heterodimer composed of a p85 regulatory subunit that contains SH2 domains, allowing it to bind to phosphorylated IRS proteins, and a p110 catalytic subunit which catalyzes the phosphorylation of 3′-OH moieties of membrane myo-inositol lipids [60]. The SH2 domain binds to phosphorylated tyrosine residues in the IRS proteins, causing activation of the p110 catalytic subunit and targeting it to the cell membrane where it preferentially phosphorylates $PI(4,5)P_2$ to form $PI(3,4,5)P_3$ [64]. This increase in $PI(3,4,5)P_3$ generates recognition sites for a number of proteins containing plekstrin homology (PH) domains, including 3-phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB/Akt) [65, 66].

Recruitment of PDK1 and PKB to the plasma membrane by $PI(3,4,5)P_3$ results in phosphorylation and activation of PKB by PDK1. Following activation, PKB is thought to mediate GLUT4 translocation by activating several substrates. Studies indicate that AS160, which contains PKB phosphorylation sites and a Rab GTPase activating protein (GAP) domain, may be involved in insulin-stimulated GLUT4 translocation [67-69]. Rab proteins have been shown to regulate vesicle budding, motility, tethering, and fusion [70]. Recently, the SNARE associated protein Synip was also identified as a PKB substrate [71]. Phosphorylation of Synip by PKB results in dissociation of the Synip–Syntaxin4 complex, allowing the assembly of a fusogenic Syntaxin4–SNAP23–VAMP2 complex necessary for GLUT4 translocation [72]. PDK1 is also responsible for activating PKCλ/ζ, which is thought to play a role in GLUT4 translocation [73-75]. Unfortunately, the mechanism of action of PKCλ/ζ in insulin-stimulated GLUT4 translocation remains

unknown, but many studies have shown that these enzymes are involved in translocation, and that they are dysfunctional in insulin-resistant states [76].

A recent study demonstrated that Cbl, another substrate for insulin receptor tyrosine kinase, is involved in a second insulin receptor signaling cascade [77]. Cbl contains multiple tyrosine residues that are phosphorylated by insulin receptor tyrosine kinase [78]. The phosphorylation of Cbl in response to insulin is dependent on the presence of the Cbl adaptor proteins APS and CAP, which are expressed in fully differentiated adipocytes [79-81]. Insulin receptor tyrosine phosphorylation causes APS to form a homodimer with the receptor, resulting in phosphorylation of APS, and providing a binding site for Cbl [82]. Once bound to APS, Cbl is phosphorylated, resulting in the translocation of GLUT4. Interestingly, insulin-stimulated Cbl phosphorylation occurs primarily in lipid rafts due to the adaptor protein CAP. CAP contains a Sorbin Homology domain that binds flotillin, a protein found in lipid rafts, and three adjacent SH3 domains that bind to the proline-rich domain of Cbl, thus recruiting Cbl to lipid rafts [77, 83, 84]. The phosphorylated tyrosine residues of Cbl provide SH2 binding sites, resulting in the recruitment of a complex of proteins containing CrkII and the guanine nucleotide exchange factor C3G, which stimulates the exchange of GTP for GDP on TC10, a Rho family small G-protein that is required for GLUT4 translocation [77, 85, 86]. The effector molecules acting downstream of TC10 that are specifically responsible for GLUT4 translocation are believed to include Exocyst complex, PKCλ/ζ, and N-WASP [87-91]. Recent studies have suggested that the actin cytoskeleton is involved in the translocation of GLUT4, and that TC10 plays an important role in the regulation of actin dynamics [87, 92-94]. In particular, N-WASP regulates actin polymerization, and induces the formation of actin comet tails on GLUT4 containing

vesicles, facilitating the translocation of these vesicles to the cell membrane [95]. The exocyst complex is a octameric complex that has been proposed to be involved in the tethering or docking of GLUT4 vesicles to the cell membrane [96].

In addition to stimulating GLUT4 translocation, insulin receptor signaling activates the mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (ERK). Insulin receptor-mediated activation of IRS proteins and/or Shc results in their interaction with the adapter protein Grb2. Grb2 recruits the Son-of-sevenless (SOS) exchange protein to the plasma membrane to induce activation of Ras with the aid of stimulation from tyrosine phosphatase SHP2, which is activated by Gab-1 or IRS1/2. Ras activation stimulates a serine kinase cascade through the stepwise activation of Raf, MEK and ERK. Once activated, ERK translocates into the nucleus and stimulates transcription factors such as $p62^{TCF}$, whose activity causes cellular proliferation or differentiation [97].

Insulin receptor activation increases glycogen stores by stimulating glucose transport and glycogen synthesis. Activated PKB phosphorylates and inactivates GSK-3 and protein kinase A (PKA), causing a decrease in the rate of phosphorylation and an increase in the activity of glycogen synthase [98]. Insulin receptor stimulation also leads to protein phosphatase 1 (PP1) activation, which dephosphorylates, and thus activates glycogen synthase [99]. Insulin inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis. In the hepatocyte, insulin stimulates the expression of glycolytic enzymes, while inhibiting the expression of gluconeogenic enzymes. More specifically, it decreases the expression of phosphoenolpyruvate carboxylase, the rate-limiting enzyme in gluconeogenesis, as well as fructose-1,6-bisphosphatase and glucose-6-phosphatase, and increases the expression of glycolytic enzymes such as glucokinase and pyruvate kinase [100].

Another anabolic effect of insulin is promoting the synthesis of lipids, and inhibiting their degradation. Studies suggest that many of these changes require an increase in the transcription factor sterol regulatory element-binding protein (SREBP)-1c, which is mediated through the activation of PKCλ/ζ [101, 102]. Insulin also inhibits lipolysis in adipocytes by preventing the phosphorylation and activation of hormone-sensitive lipase, an enzyme that hydrolyzes triacylglycerols (TAGs), freeing fatty acids and glycerol [103, 104].

1.5 Insulin Resistance and Type II Diabetes

Diabetes is a metabolic disorder resulting from a defect in insulin secretion and/or action, creating a hyperglycemic state that is responsible for disturbances in carbohydrate, lipid, and protein metabolism. The long-term complications of diabetes include retinopathy, nephropathy, neuropathy, and an increased risk of developing cardiovascular disease. Type I diabetes is a chronic autoimmune disease in which the destruction or damage of pancreatic β-cells results in insulin deficiency and hyperglycemia [105]. In contrast, type II diabetes is a common chronic disease that is characterized by a decrease in the sensitivity to the biological actions of insulin, a pathophysiological state known as insulin resistance [106, 107]. The prevalence of type II diabetes is drastically increasing and is associated with an increasing sedentary lifestyle [108]. In fact, approximately 80% of individuals who develop type II diabetes are initially obese [109]. Insulin resistance is the first clinically detectable stage in the evolution of type II diabetes [110]. Insulin resistance has been recognized since the 1930s, but it was the development of sensitive assays for insulin and quantitative methods for estimating insulin action that made it possible to define the scope of the problem and the clinical implications [111, 112].

The chronic hyperglycemia associated with type II diabetes is the result of defects in several organs. In the pancreas, insulin secretion becomes impaired due to defects in β-cells, and in the liver, glucose production increases as a consequence of increased hepatic gluconeogenesis [113]. However, prior to these defects are pathologic alterations in the response of skeletal muscle to insulin which are thought to occur decades earlier. Studies have demonstrated that the insulin resistance observed in patients with type II diabetes could be attributed to defects in insulin-stimulated muscle glycogen synthesis [114, 115]. Disruption of several important steps in the glycogen synthesis pathway could explain the defects observed in type II diabetes, including glucose uptake into the cell via GLUT 4, phosphorylation of glucose by hexokinase to glucose-6-phosphate (G6P), and the polymerization into glycogen by glycogen synthase [114]. Studies measuring the concentration of the intermediates in this pathway, as well as intracellular and extracellular glucose concentrations demonstrated that the reduced insulin-stimulated muscle glycogen synthesis that underlies insulin resistance in patients with type II diabetes is mainly attributable to reduced insulin-stimulated glucose transport into skeletal muscle cells [115, 116].

Studies in patients with type II diabetes have demonstrated an inverse relationship between fasting plasma fatty acid concentrations and insulin sensitivity, providing evidence that altered fatty acid metabolism contributes to insulin resistance in patients with type II diabetes [117]. The correlation becomes even stronger when intramyocellular lipid is considered. The association between high plasma fatty acids and insulin resistance has been recognized for decades, but only recently has the mechanism been elucidated. Due to excess circulating free fatty acids, lipid-derived metabolites begin to accumulate outside of adipose tissue depots. Hepatic accumulation of these metabolites impairs fatty

acid oxidation, causing redirection of long-chain acyl CoAs into other lipid species, such as diacylglycerols (DAGs), ceramides, and TAGs. As in the liver, intramuscular levels of lipid signaling molecules, such as long-chain acyl CoAs, DAGs and ceramides, positively correlate with TAG content and negatively correlate with insulin sensitivity [118-120]. Members of the protein kinase C family are activated by lipid-derived by-products such as DAG and these serine/threonine kinases have been shown to phosphorylate serine residues on the insulin receptor and insulin receptor substrates. Phosphorylation of these serine residues inhibits insulin receptor-mediated tyrosine phosphorylation. Inhibition of tyrosine phosphorylation on IRS proteins inhibits their ability to associate with and/or activate PI 3-kinase, resulting in acquired insulin signaling defects, decreased GLUT4 translocation and glucose uptake, and insulin resistance [121, 122]. However, lipids are not the only mediators of the effects of insulin action. The concentrations of several amino acids are elevated in patients with type II diabetes, and it has been demonstrated that increased levels of amino acids impairs skeletal muscle glucose uptake, increases hepatic gluconeogenesis and impairs insulin action [123-127]. These effects are believed to be mediated by activation of the serine/threonine protein kinases mTOR and ribosomal protein S6 kinase-1 (S6K1), resulting in the phosphorylation of IRS serine/threonine residues [123]. A recent study suggests that excess lipids and other metabolic changes that are associated with type II diabetes may trigger stress responses in the endoplasmic reticulum [128]. These changes are linked to activation of c-jun N-terminal kinases (JNK) by inositol-requiring enzyme 1 (IRE1), which phosphorylate IRS serine residues, thereby interfering with insulin action.

Obese individuals are at increased risk for developing insulin resistance and type II diabetes [12]. Different mechanisms linking obesity to type II diabetes have been

postulated, but remain poorly understood. Adipose tissue, in addition to serving an important metabolic role, is an active endocrine and paracrine organ that releases a large number of cytokines and bioactive mediators, or adipokines, which influence insulin resistance [18]. In obese individuals, changes in the secretion of adipokines that are involved in insulin sensitivity have been shown to impact the development of insulin resistance.

1.6 Adipose Tissue and Adipokines

Adipose tissue exists in various depots throughout the body including subcutaneous, gluteal, perirenal, and epididymal [129], and in two distinct forms, brown adipose tissue (BAT) and white adipose tissue (WAT), defined by its cell structure, location, color, vascularization, and function [130]. WAT functions as a primary site of energy storage as lipid droplets in adipocytes, while BAT uses large numbers of mitochondria to specialize in heat production and energy expenditure [130]. Adipose tissue contains several cell types, and can be separated into an adipocyte and a stromal-vascular fraction (SVF). The SVF contains macrophages, fibroblasts, endothelial cells, and preadipocytes which maintain the ability to differentiate into mature adipocytes for the entire human life, depending on systemic energy status and needs [131].

Adipogenesis is the process of adipocyte formation from preadipocytes, and is primarily regulated by the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ regulates adipogenesis by inducing the transcription of numerous adipogenic and lipogenic genes important for adipocyte maturation, lipid accumulation, and insulin-sensitive glucose transport, including aP2, CCAAT/enhancer-

binding protein-α (C/EBPα), perilipin, and GLUT4 [132-137]. It has been proposed that an endogenous PPARγ activator is produced during adipogenesis however, purification and identification of this ligand has been unsuccessful [138]. Many studies have demonstrated that polyunstaturated fatty acids can activate PPARγ, but PPARγ responds poorly compared to PPARα and PPARδ, suggesting that modified fatty acids may be the biological ligands [139-142]. Certain prostanoids, oxidized fatty acids, nitrated fatty acids, and lysophosphatidic acid have been shown to activate PPARγ, however the biological importance and relevance of these molecules in PPARγ biology remains unknown [143-147]. Although endogenous ligands remain elusive, the synthetic antidiabetic drugs known as thiazolidinediones (TZDs) bind directly to PPARγ and exert a number of pleiotropic effects that may play an important role in the treatment of type II diabetes[148, 149].

The classical role of adipocytes is the storage of triacylglycerides during energy consumption, and the release of fatty acids when energy expenditure exceeds energy intake. Adipocytes dramatically change size in accordance with changing metabolic needs, thus giving the adipocyte a considerable capacity for growth. During the postprandial state, lipoprotein lipase hydrolyzes triacylglycerides from very-low density lipoprotein (VLDL), and chylomicrons and their remnants. The resulting fatty acids are transported into adipocytes, and reesterified into triacylglycerides for storage [150]. When energy demand exceeds intake, triacylglycerides stored in adipocytes are hydrolyzed to glycerol and fatty acids by hormone sensitive lipase, which then participate in glucose metabolism in liver and other tissues. In addition to modulating glucose homeostasis, insulin and glucagon also regulate lipid metabolism. Insulin increases the activity and gene expression of several enzymes involved in lipogenesis, including lipoprotein lipase,

and inhibits lipolytic enzymes such as hormone sensitive lipase, resulting in an increase in fatty acid uptake and triacylglyceride synthesis [151]. However, glucagon and catecholamines induce lipolysis and promote fatty acid mobilization by increasing the activity of lipases, such as hormone sensitive lipase [152, 153].

In addition to an important metabolic role, adipose tissue is also an active endocrine organ that secretes a number of hormone like compounds, collectively termed adipokines [13, 14, 154]. The number of known adipokines has expanded rapidly, and now includes leptin, adiponectin, resistin, serpin, lipocalin-2, PAI-1, RBP4, Zn α-2 glycoprotein, vaspin, visfatin, omentin, apelin, and chemerin. Adipokines act both locally and systemically, and include pro-inflammatory cytokines and cytokine-related proteins, complement and complement-related proteins, fibrinolytic proteins, and proteins of the renin–angiotensin system, as well as a variety of other biologically active proteins with hormone-like actions. Adipokines affect adiposity, adipocyte metabolism, and inflammatory responses in adipose tissue, and also have important roles in the regulation of systemic lipid and glucose metabolism through endocrine actions in the brain, liver, and muscle. In addition to secreting proteins, adipose tissue expresses many receptors that allow it to respond to signals from traditional hormone systems as well as the central central nervous system. Although adipocytes express and secrete several adipokines, many are also derived from the SVF [16, 155].

1.7 Leptin, the Prototype Adipokine

The identification and characterization of leptin, a glycoprotein expressed and secreted by adipocytes, firmly established adipose tissue as having an important endocrine role [156]. The discovery of leptin arose from studies of mice homozygous for deleterious

mutations of the ob (obesity) or db (diabetes) genes. These mutations lead to the development of massive obesity, increased food consumption, hyperglycemia, hyperinsulinemia, insulin resistance, and impaired thermogenesis [157]. Leptin is a highly conserved 16 kDa peptide hormone predominantly produced by white adipose tissue and found in circulation and cerebrospinal fluid. Circulating leptin levels directly correlate with BMI, body fat mass, and adipocyte size, and are altered by nutritional status, falling with starvation and rising with obesity [158]. In addition, the expression of leptin can be directly upregulated by insulin [159].

Leptin crosses the blood-brain barrier and serves as an efferent signal to the central nervous system from adipose tissue. Centrally, leptin acts on specialized hypothalamic pathways, decreasing activity of neurons that stimulate appetite and inhibit energy expenditure, while simultaneously increasing activity of neurons that inhibit appetite and increase energy expenditure [160]. Leptin also increases the expression of the anorexigenic factors, pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART), and decreases the expression of the orexigenic factors, neuropeptide Y (NPY) and agouti-related peptide (AgRP) by activating its receptor OB-Rb in neurons located in the arcuate nucleus [161]. Peripherally, it has effects on skeletal muscle, liver, pancreas, adipose tissue, and numerous other cell types [160]. Leptin decreases glucose uptake into adipocytes and promotes lipolysis of triacylglycerides by inducing hormone sensitive lipase and inhibiting lipoprotein lipase [162, 163]. Leptin is also a cytokine and is structurally similar to the cytokines of the type I cytokine family, such as erythropoietin, IL-3, IL-11, IL-12, and granulocyte-colony stimulating factor [164]. The leptin receptor is also a member of the class I cytokine receptor family [165]. As a cytokine, leptin expression is increased in response to acute infection and sepsis and

directly effects $CD4^+$ T lymphocyte proliferation, macrophage phagocytosis, and secretion of inflammatory cytokines such as IL-1 and TNFα [166].

As animals and humans become obese, the role of leptin in regulating appetite, body weight, and energy homeostasis becomes more complex because leptin concentrations are already elevated due to the increased amount of leptin-secreting adipose tissue [167]. The elevated concentration of leptin induces target cells to become resistant to its actions. In obese mice an elevated leptin concentration correlates with an increased expression of suppressor of cytokine signalling (SOCS-3), a potent inhibitor of leptin signaling [168]. Reduced blood-brain barrier transport of leptin in obesity may also contribute to leptin resistance by limiting its transport into the CNS [169].

1.8 Adiponectin, an Adipocyte-Derived Insulin Sensitizer

Adiponectin is a 30 kDa secreted protein most abundantly expressed in white adipose tissue and is secreted to a similar degree from subcutaneous and visceral white adipose tissue [170-172]. Adiponectin consists of an N-terminal signal sequence, a variable domain, a collagen-like domain, and C-terminal domain. Monomeric adiponectin can trimerize to form low-molecular weight adiponectin that can combine further to 12- to 18-mers through disulfide bonds to form middle-molecular weight or high-molecular weight adiponectin. Monomeric adiponectin appears to be confined to the adipocyte, while both trimers and other oligomers are present in the circulation [173]. The function of the different adiponectin isoforms differs, depending on tissue and receptor subtype. Two receptors subtypes have been identified for adiponectin, AdipoR1 and AdipoR2. AdipoR1 is highly expressed in skeletal muscle tissue, and has lower levels of expression in brain, heart, kidney, liver, lung, spleen, and testes, and binds with high affinity globular

adiponectin [174, 175]. AdipoR2 is highly expressed in the liver and binds full-length adiponectin [175]. The large number of tissues that express adiponectin receptors suggest a wide spectrum of endocrine effects for this adipokine. In fact, this protein has been shown to play a role in whole-body metabolism, including glucose and lipid metabolism, and influences a number of diseases including obesity, type II diabetes, and cardiovascular disease [172].

Serum adiponectin levels negatively correlate with BMI, and positively correlate with insulin sensitivity, and human studies demonstrated an association between low serum adiponectin levels and obesity and type II diabetes [176-178]. Adiponectin administration to normal mice led to decreased plasma glucose levels and increased insulin sensitivity. Adiponectin mediated these changes by activating 5′-AMP-activated protein kinase (AMPK) to inhibit gluconeogenesis in the liver, PPARα and PPARγ to stimulate fatty-acid oxidation and decrease tissue triacylglyceride content in muscle and liver, and increasing GLUT4 translocation into the plasma membrane in skeletal muscle and white adipose tissue to increase glucose uptake [175, 179, 180]. Consequently, adiponectin administration to lipodystrophic or obese mice, resulted in a decrease in plasma triacylglycerides and free fatty acids, a decrease in the accumulation of lipids in skeletal muscle and liver, and a reversal of hyperglycemia and insulin resistance [175, 181, 182]. The role of adiponectin in the central regulation of energy homeostasis remains controversial [183, 184].

Similar to leptin, adiponectin also regulates several inflammatory pathways. In adipose tissue, adiponectin negatively regulates C reactive protein (CRP) and TNFα expression [185]. Adiponectin inhibits IL-8, vascular cell adhesion molecule 1 (VCAM-1) and reactive oxygen species production in endothelial cells through cAMP-PKA-

dependent signaling [176]. Adiponectin suppresses TNFα production in cardiac cells, and inhibits foam cell formation by reducing scavenger receptor A expression [185]. Lastly, in macrophages adiponectin inhibits TNFα and IL-6 production by suppressing NF-κB activation [185]. Hence, the loss of adiponectin secretion in obesity has been suggested to contribute to inflammatory responses and endothelial cell dysfunction that leads to atherosclerotic vascular changes [186, 187]. In addition, adiponectin may protect against cardiac hypertrophy in cardiac overload states such as hypertension, hypertrophic cardiomyopathy, and ischemic heart disease by inhibiting hypertrophic signals [188]. Thus, the decreased levels of the adipokine adiponectin associated with obesity represent multiple connections to cardiac risk.

1.9 Adipose Tissue and Inflammation

The SVF of adipose tissue has a composition of approximately 90% fibroblasts, endothelial cells, and preadipocytes, and 10% macrophages [189]. However, studies have shown that the number of macrophages present in white adipose tissue is directly correlated to adiposity and adipocyte size [190-192]. As individuals become obese macrophage infiltration in white adipose tissue increases, constituting up to 60% of all cells found within the tissue [191]. In adipose tissue explants from obese patients, the mRNA expression and secretion of the TNFα, IL-6, and other pro-inflammatory cytokines are elevated [193]. Adiposity also correlates with circulating levels of pro-inflammatory proteins such as CRP, IL-6, PAI-1, P-selectin, VCAM-1, fibrinogen, angiotensinogen, SAA3, and α1-acid glycoprotein, which are all produced by adipose tissue and adipocytes [194]. In addition, adipocytes express numerous receptors enabling them to sense the presence of pathogens and inflammation. Activation of these receptors

initiates multiple inflammatory signal transduction cascades that lead to the secretion of inflammatory cytokines and acute phase reactants, including TNFα, PAI-1, IL-6, macrophage migration inhibitory factor, complement factors B, D, C3, and prostaglandin E2 [16]. Further evidence supporting a connection between obesity and inflammation was demonstrated in the context of clinical weight loss studies, where weight loss caused by decreased dietary intake, increased exercise, liposuction, or bariatric surgery was associated with a decrease in circulating IL-6, CRP, PAI-1, TNFα, P-selectin, ICAM-1, VCAM-1, and IL-18 [195, 196]. Not only do these pro-inflammatory cytokines have autocrine and paracrine effects, but some adipose-derived cytokines significantly contribute to systemic energy homeostasis and inflammation.

1.10 The Pro-Inflammatory Adipokines TNFα and IL-6

TNFα is a pro-inflammatory adipokine synthesized as a 26 kDa transmembrane protein, and following enzymatic processing, gives rise to a biologically active 17 kDa soluble form [197]. In adipose tissue, TNFα is expressed primarily in the SVF, and to a lower extent in the adipocyte fraction [16]. Adipose tissue TNFα concentration is correlated with obesity, insulin resistance, fasting plasma glucose, insulin, and triacylglyceride concentrations and a reduction in body mass results in lower serum TNFα levels, and improved insulin sensitivity [193, 198, 199]. TNFα inhibits adipogenesis and lipogenesis, and increases lipolysis, resulting in a reduction in adipose tissue mass, and elevated serum lipid levels, suggesting that TNFα promotes systemic insulin resistance by increasing fatty acid metabolites in muscle and/or liver tissue [200, 201]. TNFα also directly contributes to insulin resistance by decreasing insulin-

stimulated insulin receptor autophosphorylation and subsequent IRS1 phosphorylation, resulting in a decrease in insulin-stimulated glucose uptake [202].

IL-6 is a 22 kDa pro-inflammatory adipokine synthesized and secreted from both adipocytes and SVF that regulates immune responses, cell growth and differentiation, and many metabolic processes [203, 204]. Most of adipose tissue derived IL-6 is produced by the SVF, and approximately one-third of IL-6 detected in plasma originates from adipose tissue [205]. Adipose tissue IL-6 expression and secretion increases with BMI and body fat, and plasma levels correlate with type II diabetes [193, 205, 206]. In adipose tissue and liver, IL-6 has been shown to upregulate SOCS3 expression, which impairs insulin-induced insulin receptor and IRS phosphorylation, and inhibits insulin signaling [207-209]. IL-6 also decreases adipose tissue lipoprotein lipase expression, a key enzyme in lipogenesis, casuing adipose tissue wasting and hyperlipidemia [206, 210, 211]. However, in skeletal muscle, IL-6 has been shown to increase fatty acid oxidation and glucose uptake, possibly by activating AMPK [212, 213].

The elevated levels of TNFα and IL-6 in adipose tissue in the obese state are thought to cause the decrease in circulating adiponectin levels associated with obesity. This inhibition may be mediated in part by NF-κB signaling since studies using obese diabetic mice demonstrated that inhibition of adipocyte inflammatory NF-κB signaling not only decreased cytokine levels but also increased adiponectin levels in plasma [214]. In addition, studies have shown that adiponectin inhibits activation of the transcription factor NF-κB in adipocytes and blocks the production of IL-6 and TNFα [215, 216].

Clearly obesity is associated with an increase in systemic inflammation. Intriguingly, many of the diseases associated with obesity have an important inflammatory aspect. Unfortunately, the pathologic mechanisms underlying the

connection between obesity and obesity-related diseases are not well elucidated, but an important contributing factor may be alterations in the secretion adipokines during the obese state.

Chapter II: Chemerin: At The Crossroads Of Inflammation And Obesity

2.1 Manuscript Status and Student Contribution

The figures and text presented in this chapter have been reproduced with copyright permission (Appendix I) from the review article [217]:

Ernst, M.C. and Sinal, C.J. (2010) Chemerin: At the Crossroads of Inflammation and Obesity. *Trends Endocrinol. Metab.* **21**(11): 660-7.

As first author on this review article, I wrote the article with critical evaluation from Dr. Christopher Sinal.

2.2 Abstract

Chemerin is a secreted protein with a complex, but well-established role in immune function. Parallel lines of investigation also support the notion that chemerin is a novel adipokine that regulates adipocyte development and metabolic function as well as glucose metabolism in liver and skeletal muscle tissues. A growing body of human experimental data indicates that serum chemerin levels are elevated in patients with obesity and exhibit a positive correlation with various aspects of the metabolic syndrome. Thus, the dual role of chemerin in inflammation and metabolism may provide a link between chronic inflammation and obesity as well as obesity-related disorders such as type II diabetes and cardiovascular disease.

2.3 Discovery

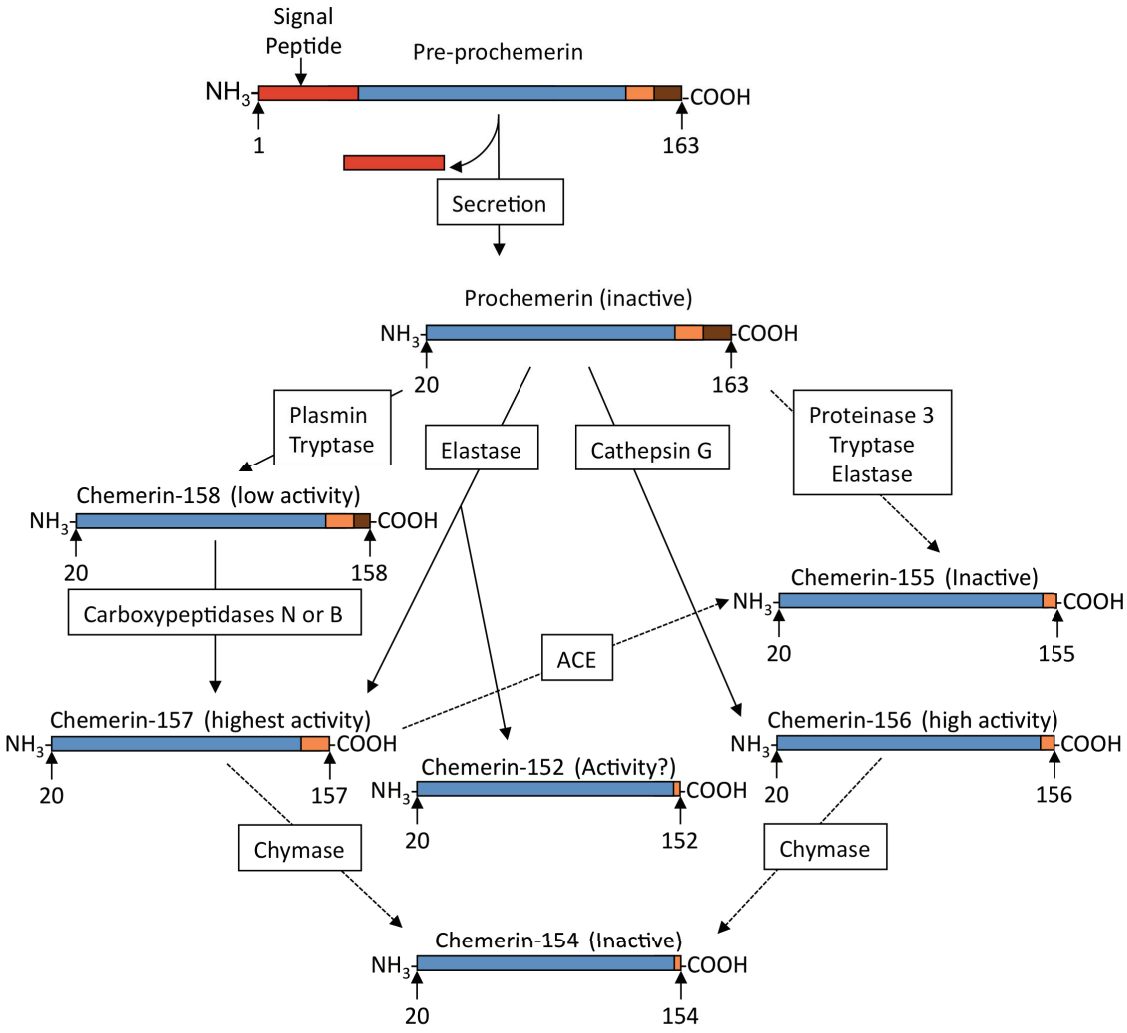
Chemerin, also known as tazarotene-induced gene 2 (TIG2) or retinoic acid receptor responder 2 (RARRES2), was originally identified as a novel retinoid-responsive gene in psoriatic skin lesions [218]. The first evidence of a biological function for chemerin came later with a report identifying this protein as secreted ligand of the orphan G protein-coupled receptor chemokine-like receptor 1 (CMKLR1) [219]. Human chemerin and CMKLR1 share 72 % and 82 % nucleotide sequence homology with their mouse counterparts respectively, and it has been shown that human chemerin activates mCMKLR1 [220]. More recent studies have demonstrated that chemerin also serves as a ligand for at least two additional receptors including chemokine (C-C motif) receptor-like 2 (CCRL2) and G protein-coupled receptor 1 (GPR1). CCRL2 appears to be a non-signaling receptor that binds chemerin and increases the local concentration of the peptide [221, 222]. GPR1 is involved in nutrient sensing in yeast, but studies examining its role in

mammals are currently lacking [223, 224]. The majority of studies to date have described the physiological functions of chemerin elicited through activation of CMKLR1. Very little is known regarding the CMKLR1 signaling cascade, however studies have shown that CMKLR1 activation results in intracellular calcium release, inhibition of cAMP accumulation, and phosphorylation of p42-p44 MAP kinases, through the G_i class of heterotrimeric G proteins [219]. Various cell types involved in innate and adaptive immunity express CMKLR1, and chemerin is now known to function as a chemoattractant that promotes the recruitment of these cells to lymphoid organs and sites of tissue injury [219, 225-227]. In a parallel line of research, chemerin expression and secretion has been shown to increase dramatically with adipocyte differentiation [228, 229]. Moreover, loss of chemerin or CMKLR1 expression almost completely abrogates adipogenesis in cell-based models, and modifies the expression of genes important in glucose and lipid metabolism, including GLUT4, DGAT2, leptin, and adiponectin [228]. This work inspired the current hypothesis that chemerin is a novel adipokine and established a new area of study for this protein.

2.4 Structure and Processing

Chemerin is translated as a 163 amino acid pre-propeptide that is secreted as a 143 amino acid (18 kDa) propeptide following proteolytic cleavage of a signal peptide [219, 230] (Figure 4). This propeptide has low biological activity and requires further extracellular C-terminal processing by plasmin, carboxypeptidases, or serine proteases of the coagulation, fibrinolytic, and inflammatory cascades [219-221, 226, 230]. Interestingly, the extent of C-terminal cleavage depends on the location from which

Figure 4. Proteolytic Processing of Chemerin. Chemerin is produced as a pre-proprotein, pre-prochemerin (1–163), which requires N-terminal cleavage of a secretion signal peptide before it is secreted as an inactive precursor protein, prochemerin (20–163). Extracellular proteolytic processing of the carboxyterminus of prochemerin exposes the bioactive region. Cathepsin G cleaves seven C-terminal amino acids from prochemerin (chemerin-156), elastase is able to cleave six (chemerin-157), eight (chemerin-155) or eleven (chemerin-152), plasmin cleaves five (chemerin-158), and tryptase cleaves five (chemerin-158) or eight (chemerin-155). Multiple cleavages might be required to fully activate chemerin, with an initial tryptase cleavage resulting in chemerin with low activity (chemerin-158), and a second cleavage by carboxypeptidase N or B producing highly active chemerin (chemerin-157). Chemerin-156 and -157 activities are terminated by chymase cleavage to produce inactive chemerin-154. Chemerin-157 activity might also be terminated by ACE cleavage to produce inactive chemerin-155. The number (e.g. -157) refers to the terminal amino acid position of the processed protein.


Signal Peptide
Pre-prochemerin
NH3
COOH
1
163
Secretion
Prochemerin (inactive)
NH3
COOH
20
163
Plasmin
Tryptase
Elastase
Cathepsin G
Proteinase 3
Tryptase
Elastase
Chemerin-158 (low activity)
NH3
COOH
20
158
Carboxypeptidases N or B
Chemerin-155 (Inactive)
NH3
COOH
20
155
ACE
Chemerin-157 (highest activity)
NH3
COOH
20
157
Chemerin-152 (Activity?)
NH3
COOH
20
152
Chemerin-156 (high activity)
NH3
COOH
20
156
Chymase
Chymase
Chemerin-154 (Inactive)
NH3
COOH
20
154

chemerin is isolated. For example, chemerin from human ovarian ascites fluid, serum, and hemofiltrate lacks six, eight, and nine C-terminal amino acids, respectively [231, 232]. These findings are consistent with observations that several enzymes are capable of processing chemerin to an active form. *In vitro* studies have shown that cathepsin G cleaves seven C-terminal amino acids from prochemerin, elastase is able to cleave six, eight, or eleven, plasmin cleaves five, and tryptase cleaves five or eight [225, 232] (Figure 4). In some cases, multiple cleavages are required to fully activate chemerin. For example, an initial tryptase cleavage at amino acid 158 results in chemerin with very low activity. However, this product serves as a substrate for a second cleavage by carboxypeptidase N or B producing fully activated bioactive chemerin [233] (Figure 4). Proteolytic processing is also believed to be involved in the inactivation of chemerin. In particular, neutrophil-derived serine protease proteinase, mast cell chymase, and angiotensin converting enzyme have been shown to convert bioactive forms of chemerin to inactive derivatives [234, 235] (Figure 4). Thus, proteolytic processing of chemerin is a key regulatory mechanism that may determine both systemic and local concentrations of bioactive chemerin. As such, a key area of research is to elucidate the physiologic and pathophysiologic relevance of chemerin processing. Interestingly, many of the known biological activities of chemerin can be largely recapitulated by small peptides that are identical or synthetic variations of the 9-15 C-terminal amino acids of chemerin 20-157 [236-239]. While an unlikely proposition, this suggests that the remaining portion of the protein is dispensable and devoid of function. Further experimentation is required to determine if the N-terminal region is involved in the formation of multimeric complexes of chemerin or in the interaction of chemerin with GPR1 and CCRL2.

2.5 Inflammation and Obesity

The first recognized function of chemerin, acting through CMKLR1, was to promote chemotaxis of immature dendritic cells (DCs) and macrophages [219]. It is now known that CMKLR1 is expressed in a number of immune cells, including immature plasmacytoid DCs, myeloid DCs, macrophages, and NK cells [220, 226, 240], and that serum chemerin levels correlate with levels of the pro-inflammatory cytokines TNFα, IL-6, and CRP [241, 242]. Pathologically, chemerin is expressed in psoriatic lesions [218], and several effector cells of the innate immune system that are influenced by chemerin, including DCs, monocytes, macrophages and NK cells have been implicated in the pathogenesis of psoriasis [243-245]. Chemerin expression is believed to be a marker for the early phase of developing lesions and is also thought to promote the recruitment and activation of plasmacytoid DCs [243, 246-248]. This suggests that chemerin and CMKLR1 are involved in the recruitment of various immune cells into the lesion and may influence the crosstalk between different cell types responsible for controlling the initiation and progression of psoriatic inflammation [249-251]. Chemerin and CMKLR1 appear to play important roles in other autoimmune states such as experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis. In this model, CMKLR1 null mice developed less severe clinical and histologic disease, and had lower CNS inflammation relative to control mice [252]. Since macrophages play a critical role in propagating the inflammatory cascade, a loss of CMKLR1-dependent recruitment of these cells by chemerin could account for the observed inflammatory differences.

While much experimental evidence supports a pro-inflammatory role for chemerin/CMKLR1, other studies suggest that this signalling pathway may have an anti-inflammatory function. For example, a study by Luangsay et al. [253] utilizing an LPS-

induced mouse model of acute lung inflammation demonstrated that chemerin treatment simultaneously increased the mobilization of airway macrophages and decreased neutrophil recruitment and activation, suggesting both a pro-inflammatory and anti-inflammatory role for chemerin and CMKLR1. These chemerin-induced effects were not observed in CMKLR1 null mice [253]. Interestingly, CMKLR1 null mice treated with LPS alone exhibited significantly greater neutrophil and macrophage recruitment in lung tissue compared to wildtype mice, indicating the importance of CMKLR1 as an anti-inflammatory mediator. Moreover, in a zymosan-induced mouse model of peritonitis, mice treated with a synthetic 15-mer C-terminal chemerin peptide exhibited significantly less neutrophil and monocyte recruitment, and a decrease in pro-inflammatory cytokine expression [237]. The absence of this effect in CMKLR1 null mice supported that the anti-inflammatory effects of chemerin were elicited through CMKLR1 [237]. However, it is important to note that a second independent study did not recapitulate key aspects of this study and failed to detect CMKLR1 activation by the same synthetic peptide using an aequorin-based calcium release assay [253]. Therefore, the ability of this chemerin derivative to act as an anti-inflammatory peptide requires further investigation.

In recent years it has become clear that obesity is commonly associated with chronic low-grade systemic inflammation. In addition to adipocytes, adipose tissue contains a number of lymphocytes located in the stromal vascular fraction, including macrophages, NK cells, and T cells [192, 254, 255]. As individuals become obese and their adipocytes enlarge, adipose tissue undergoes molecular and cellular alterations affecting systemic metabolism and inflammation. Serum levels of the inflammatory markers TNFα, IL-6, and CRP are elevated in obesity [193, 194], and adipose tissue appears be a substantial source of these cytokines [256]. Furthermore, obesity correlates

with a significant increase in macrophage infiltration in white adipose tissue, with this cell type constituting up to 60 % of all cells found in obese adipose tissue [192, 254]. These infiltrated macrophages produce pro-inflammatory cytokines that can exacerbate insulin resistance by interfering with insulin signaling [256]. Intriguingly, the ability to promote chemotaxis of immature DCs and macrophages through activation of CMKLR1 was among the first recognized functions of chemerin [219]. Presently there is no empirical evidence linking chemerin/CMKLR1 signaling with inflammation of adipose tissue, however data from the aforementioned studies exploring the immune functions of chemerin make this a plausible proposition. CMKLR1 is expressed in a number of immune cell types known to accumulate in obese adipose tissue, including immature plasmacytoid DCs, myeloid DCs, macrophages, and NK cells [220, 226, 240], and activation of CMKLR1 by chemerin may promote recruitment of these cells to dead and dying adipocytes found in obese adipose tissue [257, 258]. Moreover, as discussed in the next section, white adipose is a major site of chemerin expression, and the secretion of chemerin from adipose tissue increases with adipocyte differentiation and obesity.

2.6 Metabolism and Obesity

In addition to having an important energy storage function, white adipose tissue serves as an active endocrine organ that secretes a number of hormone-like compounds, collectively termed adipokines [13, 14, 154]. Adipokines include pro-inflammatory cytokines and related proteins, complement and complement-related proteins, proteins of the fibrinolytic cascade, vasoactive proteins and other biologically active peptides with hormone-like actions. Adipokines affect adiposity, adipocyte metabolism, and inflammatory responses in adipose tissue and have important roles in systemic lipid and

glucose metabolism. In 2007 it was first reported that both chemerin and CMKLR1 were highly expressed in white adipose tissue from mouse, rat, and human samples, identifying chemerin as a novel adipokine with potential autocrine and paracrine functions [228, 229]. In addition to this high level of expression, white adipose tissue likely also possesses a substantial capacity to bioactivate chemerin. For example, cathepsin G [259] and tryptase [260] are expressed in adipose tissue, suggesting that chemerin-155, -156, and -158, all bioactive pro-inflammatory forms of chemerin, can be produced in this tissue. Cathepsin S, thought to produce an anti-inflammatory chemerin derivative, is also expressed in adipose tissue [261]. Lastly, the expression of chymase and ACE in adipose tissue [262-264] suggests that bioactive forms of chemerin can be inactivated in adipose tissue. A key area of research will be to identify the forms of chemerin produced in adipose tissue and any alterations that may occur as a consequence of obesity. This would provide valuable insight into the physiologic and pathophysiologic influence of white adipose on both local and systemic levels of bioactive chemerin.

Chemerin expression and secretion increases dramatically with adipogenesis, and loss of chemerin or CMKLR1 expression in preadipocytes severely impairs differentiation into mature adipocytes [228, 265]. Beyond this autocrine function in adipocytes, chemerin/CMKLR1 signaling may have paracrine functions within adipose tissue. As proposed in the previous section, chemerin serves as a chemoattractant for various types of immune cells and may thereby contribute to white adipose tissue inflammation with obesity. Additionally, adipose is a highly vascularized tissue, and inhibiting angiogenesis has been shown to prevent adipose tissue growth and the development of obesity, diabetes, and cardiovascular disease [266-268]. CMKLR1 is expressed in human endothelial cells and is upregulated by the pro-inflammatory

cytokines TNFα, IL-6, and IL-1β [269]. Studies have also shown that chemerin activates key angiogenic pathways and induces angiogenesis *in vitro* [269, 270]. Therefore, the elevated expression and secretion of chemerin during adipogenesis could also support adipose tissue growth by inducing angiogenesis and increasing adipose tissue vascularization.

The bulk of human data supports a linkage between chemerin, obesity and metabolic syndrome - a cluster of metabolic disorders that increase the risk for diabetes and cardiovascular disease. For example, a study of a Mexican-American population reported significantly higher serum chemerin levels in type II diabetic patients compared to normoglycemic controls, as well as in obese and overweight subjects compared to lean controls [271]. Plasma chemerin levels correlated positively with BMI, fasting glucose, fasting serum insulin, plasma triacylglycerides, and total serum cholesterol, and a negative correlation with HDL-cholesterol [271]. A separate study of a Mauritian population of mixed ethnicity demonstrated that after adjusting for gender and age, serum chemerin levels were significantly elevated in overweight and/or obese subjects, and positively correlated with waist circumference, waist-to-hip ratio, homeostasis model assessment of insulin resistance (HOMA-IR), and triacylglycerides, and negatively correlated with HDL [229] Other studies have similarly reported higher chemerin levels in type II diabetic and obese patients, as well as a positive correlation between serum chemerin levels and leptin, resistin and CRP, TNFα and IL-6 [241, 242]. A study of a Caucasian population found that individuals with metabolic syndrome had significantly higher chemerin serum levels compared to healthy subjects, and positive correlations were observed between serum chemerin and glucose, triacylglycerides, systolic and diastolic blood pressure [272]. In addition, selecting a serum chemerin concentration

threshold of 240 ug/L allowed the researchers to diagnose metabolic syndrome with a sensitivity of 75 % and specificity of 67 % [272]. Despite the considerable evidence linking circulating chemerin levels with adiposity and various aspects of the metabolic syndrome, we are unaware of any genome wide analyses identifying the genes encoding chemerin or any cognate receptors as candidate susceptibility loci for human disease. However, a recent genome-wide association study [270] reported that serum chemerin levels are highly heritable and found that the single nucleotide polymorphism (SNP) showing the strongest evidence of associated with plasma chemerin levels was located in the EIDL3 gene, which has a known role in angiogenesis. At present, targeted genetic studies of chemerin and CMKLR1 are very limited. The only example in the current scientific literature reported that while SNPs of the chemerin gene were not associated with total adiposity, there was an association with increased visceral fat mass in lean subjects [273]. These data suggest an influence of chemerin on regional fat distribution and in particular, visceral adiposity, which is most strongly associated with the metabolic derangements that can occur with obesity [274].

The aforementioned studies clearly identify a relationship between serum chemerin levels and obesity. However, as chemerin is expressed in anatomical sites in addition to adipose, most notably the liver, the source of elevated chemerin levels remains to be definitively established. A recent analysis of portal, hepatic and systemic venous blood chemerin levels in humans indicated similar chemerin levels in the portal and systemic vein, suggesting that visceral adipose tissue is not a major contributor to serum chemerin levels [241]. However, chemerin levels were higher in hepatic vein blood samples, indicating that chemerin is synthesized and secreted by the liver [241]. In a separate study, serum chemerin levels in patients that had undergone bariatric surgery for

the purpose of weight loss were significantly reduced post-surgery and correlated with BMI and fat mass [275, 276]. Examination of chemerin in women with polycystic ovary syndrome (PCOS), a common endocrinopathy associated with insulin resistance, pancreatic β-cell dysfunction, impaired glucose tolerance, type II diabetes, dyslipidemia, and visceral obesity revealed that both chemerin mRNA and protein levels are elevated in subcutaneous and omental adipose tissue from PCOS patients [277]. In addition, chemerin secretion from female adipose tissue explants revealed a significantly higher release of chemerin from obese versus lean subjects [278]. Recent animal studies reported the parallel findings that obese and diabetic mice have elevated circulating levels of chemerin [279, 280]. Thus, while not unequivocal, the balance of experimental evidence suggests that chemerin is secreted from both liver and adipose tissue, but that the latter is a dynamic and modifiable source of chemerin in obesity.

While evidence exists for a linkage between circulating chemerin levels and aspects of metabolic syndrome, other studies suggest that local concentrations may be a more important determinant of pathologic outcomes such as cardiovascular disease. For example, serum chemerin levels were reported to be only weakly correlated with coronary plaque burden and the number of non-calcified plaques in humans [242]. Moreover, following adjustment for established cardiovascular disease risk factors, these correlations were no longer present. However, another study demonstrated that aortic and coronary atherosclerosis was positively correlated with chemerin expression in periaortic and pericoronary adipose tissue, respectively [281]. This suggests that locally produced chemerin affects the development of atherosclerosis in a paracrine manner [281]. Thus, while serum chemerin levels do not predict coronary atherosclerosis, local chemerin concentrations may influence plaque development. Atherosclerosis is a progressive

inflammatory disease, and the accumulation of macrophages in atherosclerotic plaques positively correlates with disease progression. A potential explanation is that increased local chemerin concentrations in coronary vessels promote macrophage recruitment and influence inflammatory response in atherosclerotic plaques. Again, future studies are required to elucidate the role of chemerin in inflammation and plaque initiation and progression.

While it is clear that serum chemerin levels are elevated in obesity, the mechanisms regulating chemerin expression remain poorly understood. Insulin has been shown to increase chemerin secretion from adipose tissue both dose and time dependently *in vitro* and in tissue explants [277]. IL-1β, a pro-inflammatory cytokine associated with insulin resistance, induces chemerin mRNA expression and secretion dose-dependently from 3T3-L1 derived adipocytes [282]. TNFα, another pro-inflammatory cytokine associated with insulin resistance, also increases serum chemerin levels *in vitro* as well as chemerin synthesis and secretion from 3T3-L1 adipocytes [280]. An induction in expression caused by pro-inflammatory cytokines suggests that NFκB may modulate chemerin mRNA expression [282]. These findings are supported by the fact that hyperinsulinemia and elevated pro-inflammatory cytokine levels are commonly associated with obesity. Other studies have examined the relationship between PPARγ activation by TZDs and chemerin expression, with some studies suggesting that PPARγ activation or expression is associated with elevated chemerin expression [265, 283-285]. In contrast, TZD activation of PPARγ in mature adipocytes has been reported to reduce chemerin expression [284, 286]. These conflicting results suggest that further studies are required to determine the role of PPARγ in regulating chemerin expression, particularly given the importance and common use of TZDs as a treatment for type II diabetes.

In summary, the majority of clinical data shows that serum chemerin levels are correlated with body fat, glucose and lipid metabolism, and inflammation, suggesting that this adipokine plays a role in the pathophysiology of obesity and metabolic syndrome. While correlative studies provide meaningful information, they do not determine the function of chemerin in obesity and metabolic syndrome, or determine whether excess chemerin increases adiposity and disrupts metabolic function or if elevated chemerin levels are a consequence, or compensatory response during and following the development of obesity and its comorbidities. Therefore, further studies are required to examine the relationship between serum chemerin levels, obesity, and facets of the metabolic syndrome.

2.7 Glucose Homeostasis

Obesity is an established risk factor for insulin resistance and type II diabetes, and alterations in adipokine secretion in obesity are believed to play a significant role in the development of these metabolic disorders [1, 12, 16, 287-290]. The elevated serum chemerin levels observed in humans and mice suggest that chemerin may also influence the dysregulation of glucose metabolism that often occurs with obesity. However it is important to note that hyperinsulinemia, which is commonly found in obese and type II diabetic patients, has been reported to increase serum chemerin levels [277]. *In vitro* studies using 3T3-L1 adipocytes have provided conflicting results, with one study reporting decreased insulin-stimulated glucose uptake [291] and another showing increased insulin-stimulated glucose uptake and IRS1 tyrosine phosphorylation following chemerin treatment [282]. However, the methodology of these studies differed in a number of respects. For example, the study reporting increased glucose uptake treated

3T3 adipocytes with approximately 6 nM chemerin for 12 h in serum containing media before measuring insulin stimulated glucose uptake [291]. The study that observed a decrease in glucose uptake treated the adipocytes with 10 μM chemerin for 49 h in serum free media before measuring glucose uptake [282]. Thus, the different concentrations as well as treatment duration and conditions may have contributed to the discrepant results. For example, the shorter, lower dose treatment may have caused an acute increase in glucose uptake, while the longer, higher dose treatment may have resulted in a negative feedback response, or potentially the establishment of a resistant state that produced a net decrease of glucose uptake. In another study, treatment of primary human skeletal muscle cells with 60 nM chemerin for 24 h resulted in an increase in phosphorylation of an IRS1 serine residue known for negatively modulating the actions of insulin coincident with a decrease of insulin-stimulated glucose uptake [278]. A concomitant decrease of Akt, GSK3α and GSK3β phosphorylation was also observed. In mice, as described in detail in Chapter III, chemerin treatment exacerbates glucose intolerance in obese/diabetic (db/db), but not normoglycemic models, by decreasing serum insulin levels, reducing adipose tissue glucose uptake, and causing a significant decrease in liver and total tissue glucose uptake [279]. Similarly, overexpression of chemerin has been shown to exacerbate glucose intolerance by reducing insulin-stimulated Akt1 phosphorylation and activation of 5'AMP-activated protein kinase in the skeletal muscle [292]. Chemerin-induced dysregulation of glucose uptake in adipocyte and myocyte cultures suggests an insulin-dependent GLUT4 mechanism, while a decrease in serum insulin levels and liver glucose uptake in obese/diabetic (db/db) mice suggests an insulin-independent GLUT2 mechanism. Thus, the mechanisms by which chemerin alters glucose homeostasis remain

unknown and these conflicting findings illustrate a need to clarify the role of chemerin in glucose metabolism.

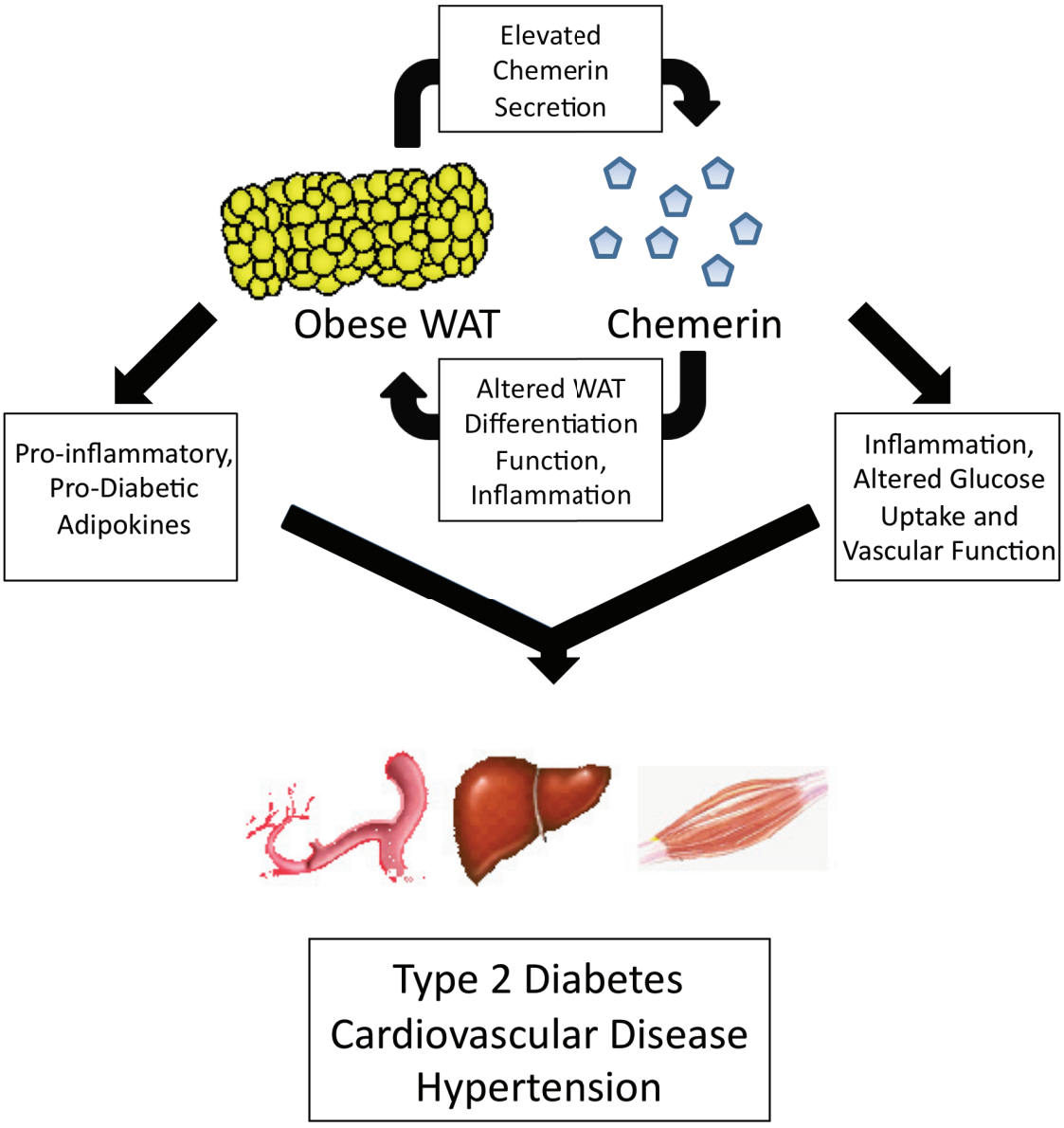
2.8 Summary and Concluding Remarks

Experimental evidence supports a role for chemerin in various aspects of human physiology/pathophysiology including obesity, inflammation, and insulin resistance. The majority of human data indicates that chemerin is elevated in obesity/diabetes, and that the source of elevated serum chemerin levels is adipose tissue (Figure 5). However, the precise role of chemerin in these disorders remains unclear. Since the initial identification of chemerin as ligand for CMKLR1 [219], a tremendous amount of information has emerged regarding this protein. While much remains to be learned regarding the physiologic/pathophysiologic relevance of chemerin, two parallel themes have clearly emerged – inflammation and metabolism. Given the ever increasing evidence for these dual roles, chemerin provides a plausible linkage between chronic inflammation and the frequent association of this condition with obesity and obesity-related diseases. As such, further studies of chemerin and cognate receptors will provide valuable insight and have the potential to identify novel therapeutic approaches to these prevalent disorders.

2.9 Thesis Proposal

Obesity, characterized by an excess of adipose tissue, is an established risk factor for the development of insulin resistance and type II diabetes. Different mechanisms linking obesity with these comorbidities have been postulated, but remain poorly understood. Adipose tissue secretes a number of hormone-like compounds, termed adipokines that are important for the maintenance of normal glucose metabolism.

Figure 5. Obesity, Inflammation and Chemerin. The secretion of chemerin from white adipose tissue is elevated in obesity. Chemerin from white adipose tissue promotes adipocyte differentiation, alters adipose tissue function and might play a role in angiogenesis, a process essential for the expansion of white adipose tissue. Chemerin is also a pro-inflammatory adipokine, causing an increase in secretion of pro-inflammatory and prodiabetic adipokines, which further impair adipose tissue metabolic function and have negative systemic effects including impaired insulin sensitivity, altered glucose and lipid metabolism, and decrease in vascular function in other tissues. The dual role of chemerin in inflammation and metabolism suggests that it is involved in the crosstalk that integrates the inflammatory response with metabolism in obesity. The resulting changes in metabolic homeostasis and vascular function might set the stage for the development of T2DM, cardiovascular disease and hypertension.


Elevated Chemerin Secretion
Obese WAT
Chemerin
Altered WAT Differentiation Function, Inflammation
Pro-inflammatory, Pro-Diabetic Adipokines
Inflammation, Altered Glucose Uptake and Vascular Function
Type 2 Diabetes
Cardiovascular Disease
Hypertension

Alterations in the secretion of adipokines with obesity are believed to contribute to the undesirable changes in glucose metabolism that ultimately result in the development of type II diabetes. Chemerin is a recently discovered secreted protein with a role in adaptive and innate immunity [219, 225-227]. The first recognized function of chemerin, acting through the G protein-coupled receptor (GPCR) chemokine-like receptor 1 (CMKLR1), was to promote chemotaxis of dendritic cells and macrophages [219]. Subsequently, chemerin and CMKLR1 were shown to be highly expressed in WAT [228, 229]. Chemerin expression and secretion increases dramatically with adipogenesis, and loss of chemerin or CMKLR1 in preadipocytes severely impairs adipocyte differentiation [228, 265].

Several studies have now investigated the effect of chemerin on glucose uptake. Whether chemerin stimulates or inhibits glucose uptake in 3T3-L1 adipocytes *in vitro* is controversial [282, 291]. Treatment of primary human skeletal muscle cells with chemerin decreases insulin-stimulated glucose uptake [278]. The expression of chemerin and its three receptors in tissues central to glucose homeostasis indicates that changes in the biological actions of chemerin may contribute to disruptions in glucose metabolism that occur with obesity. Therefore, we have proposed that chemerin contributes to the pathology of insulin resistance through the regulation and modulation of glucose homeostasis in white adipose, skeletal muscle, and liver tissue. Furthermore, we hypothesize that the loss of the chemerin receptor CMKLR1 *in vivo* would reduce adiposity and impact glucose homeostasis.

Chapter III: Chemerin Exacerbates Glucose Intolerance in Mouse Models of Obesity and Diabetes

3.1 Manuscript status and Student Contribution

The figures and text presented in this chapter have been reproduced with copyright permission (Appendix I) from the article [279]:

Ernst, M.C., Issa, M., Goralski, K.B. and Sinal C.J. (2010) Chemerin exacerbates glucose intolerance in mouse models of obesity and diabetes. *Endocrinology* **151**(5): 1998-2007.

As first author on this article, I performed all of the experiments, analysis, data interpretation, and statistics with technical assistance from Mark Issa. I wrote the manuscript with critical evaluation from Dr. Christopher Sinal and Dr. Kerry Goralski.

3.2 Abstract

Obesity, characterized by an excess of adipose tissue, is an established risk factor for cardiovascular disease and type II diabetes. Different mechanisms linking obesity with these comorbidities have been postulated, but remain poorly understood. Adipose tissue secretes a number of hormone-like compounds, termed adipokines that are important for the maintenance of normal glucose metabolism. Alterations in the secretion of adipokines with obesity are believed to contribute to the undesirable changes in glucose metabolism that ultimately result in the development of type II diabetes. In the present study, we have shown that serum levels of the novel adipokine chemerin are significantly elevated in mouse models of obesity/diabetes. The expression of chemerin and its receptors, chemokine-like receptor 1, chemokine (C-C motif) receptor-like 2, and G protein coupled receptor 1 are altered in white adipose, skeletal muscle, and liver tissue of obese/diabetic mice. Administration of exogenous chemerin exacerbates glucose intolerance, lowers serum insulin levels and decreases tissue glucose uptake in obese/diabetic, but not normoglycemic mice. Collectively, these data indicate that chemerin influences glucose homeostasis and may contribute to the metabolic derangements characteristic of obesity and type II diabetes.

3.3 Introduction

Obesity, characterized by an excess of adipose tissue, has reached epidemic proportions worldwide [11]. Obese individuals are at increased risk for hypertension, dyslipidemia, cardiovascular disease, and type II diabetes [1, 12, 287]. A major factor underlying the adverse metabolic consequences of obesity is believed to be insulin resistance [106, 107]. Adipose tissue serves as an active endocrine organ that secretes a

number of adipokines that affect adiposity, adipocyte metabolism, and inflammatory responses in adipose tissue, and have important roles in the regulation of systemic glucose metabolism [13-15, 154, 228, 293, 294]. Serum levels of many adipokines are affected by the degree of adiposity and decreased insulin sensitivity associated with obesity may reflect an imbalance in the secretion of pro-inflammatory/pro-diabetic and anti-inflammatory/anti-diabetic adipokines that occur as a consequence of the dysfunctional adipose tissue that develops with obesity [16, 288-290, 295-299].

Chemerin is a novel adipokine that has a role in adaptive and innate immunity, and regulates adipocyte differentiation and metabolism by binding to and activating the GPCR CMKLR1 [219, 226, 228, 230, 283]. Chemerin also serves as a ligand for CCRL2 and GPR1. Chemerin is secreted as an 18 kDa inactive proprotein that can be rapidly converted by C-terminal proteolytic cleavage into its active 16 kDa form [219, 220, 226, 230, 232]. Previously we have reported that loss of *chemerin* or *CMKLR1* expression in 3T3-L1 preadipocytes severely impairs differentiation into mature adipocytes and reduces the expression of genes involved in glucose and lipid metabolism [228]. Takahashi *et al.* reported that recombinant mouse chemerin modestly increased insulin-stimulated tyrosine phosphorylation of IRS-1 and glucose uptake in 3T3 adipocytes [291]. In contrast, Kralisch *et al.* reported that chemerin significantly decreased insulin-stimulated glucose transport in 3T3 adipocytes [282, 291]. Similarly, Sell *et al.* reported that chemerin reduces glucose uptake in human skeletal muscle cells at the level of IRS1 and Akt [278]. These findings illustrate the need to clarify the role of chemerin in glucose metabolism.

Recent clinical studies have demonstrated that serum chemerin levels are elevated in obese patients compared to healthy patients. These cases reported positive correlations between serum chemerin levels and BMI, serum triacylglycerides, and blood pressure

[229, 271, 272]. The expression of chemerin and its three receptors in tissues central to glucose homeostasis indicates that changes in the biological actions of chemerin may contribute to disruptions in glucose metabolism that occur with obesity. Therefore, we have proposed that chemerin contributes to the pathology of insulin resistance through the regulation and modulation of glucose homeostasis in white adipose, skeletal muscle, and liver tissue. To investigate this, we examined the expression of chemerin and the cognate receptors in murine models of obesity and diabetes. Furthermore we also tested the effect of chemerin on blood glucose and insulin levels in these models.

3.4 Materials and Methods

3.4.1 Animal Protocol and Housing

All protocols and procedures were approved by the Dalhousie University Committee on Laboratory Animals and are in accordance with the Canadian Council on Animal Care guidelines. $Lep^{ob/ob}$ (ob/ob), $Lepr^{db/db}$ (db/db), and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Low fat (LF) diet containing 10 kcal% fat (D12450B,) and high fat (HF) diet containing 45 kcal% fat (D12451) were purchased from Research Diets (New Brunswick, New Jersey). The fat source in the diets was derived from lard (LF 4 %, HF 39 %) and soyabean oil (LF 6 %, HF 6 %). Mice were housed in groups of two to five in filter-top cages with a fixed 12 h light:12 h dark cycle. $Lep^{ob/ob}$, $Lepr^{db/db}$, and C57BL/6 littermates were fed standard mouse chow (Prolab RMH 3000, PMI Nutrition International, Inc., St. Louis, Missouri). At 13 weeks of age, ob/ob, db/db, and C57BL/6 mice were anaesthetized with an intraperitoneal (IP) injection of 80 mg/kg sodium pentobarbital (CMTC Pharmaceuticals, Cambridge, Ontario). Blood was collected by cardiac puncture, allowed to clot for 2 h at room temperature to achieve

maximum chemerin activity [226], and then centrifuged; serum was stored at -80^{o}C until used. Liver, skeletal muscle (gastrocnemius), and epididymal white adipose were snap frozen in liquid nitrogen prior to RNA extraction. C57BL/6 mice used in the diet-induced obesity (DIO) experiments were switched to the LF or HF diet at 6 weeks of age for a duration of 18 weeks. DIO mice and C57BL/6 controls were anaesthetized at 25 weeks of age.

3.4.2 RNA Isolation and Quantification

Liver RNA was isolated using Trizol® Reagent (Invitrogen, Burlington, Ontario), and white adipose and skeletal muscle RNA were isolated using RNeasy Mini Kits (Qiagen, Mississauga, Ontario) as per the manufacturer's instructions. To quantify RNA, samples were diluted in RNase free water, placed in a UV spectrophotometric plate, and the absorbance at 260 and 280 nm measured using a PowerWave$_x$ spectrophotometer plate reader (Bio-Tek Instruments, Winooski, Vermont). The quantity of RNA was calculated using Beer's law with an extinction coefficient of 40 μg/mL.

3.4.3 Reverse Transcription and Quantitative Real-Time PCR

From the isolated RNA samples, 0.5 μg of RNA was reverse transcribed using AffinityScript Reverse Transcriptase (Stratagene, La Jolla, California) as per the manufacturer's instructions, and 1 μL of cDNA product was amplified by quantitative real-time PCR. All genes were normalized to mouse *cyclophilin A* expression. Primer sequences are shown in Table 1. To measure gene expression, 1 μL of reverse transcription product was combined with 19 μL of a master mix containing Brilliant® SYBR® Green QPCR 2X Master Mix (Stratagene, La Jolla, California), Rox reference

Table 1. Quantitative Real-Time PCR Primer Sequences.

Gene	Accession Number	Sequence (5' -> 3')
Cyclophillin A	X52803.1	Fw GAG CTG TTT GCA GAC AAA GTT C
		Rv CCC TGG CAC ATG AAT CCT GG
Chemerin	NM_027852	Fw TAC AGG TGG CTC TGG AGG AGT TC
		Rv CTT CTC CCG TTT GGT TTG ATT G
CMKLR1	NM_0081153	Fw CAA GCA AAC AGC CAC TAC CA
		Rv TAG ATG CCG GAG TCG TTG TAA
CCRL2	NM_017466	Fw CTC TGC TTG TCC TCG TGC TT
		Rv GCC CAC TGT TGT CCA GGT AG
GPR1	NM_146250.1	Fw CAC CTT TCG GGG TGT CAT T
		Rv AAG GAA ATG TGT TAA TGT TCT G

dye (Stratagene, La Jolla, California), water, and gene-specific primers (2.5 μM). The Mx3000P® Thermocycler was programmed with cycling conditions consisting of 10 min at 95 ^{o}C for initial denaturation followed by 40 cycles of 95 ^{o}C for 20 s, 60 ^{o}C for 18 s, and 72 ^{o}C for 30 s for denaturation, annealing, and polymerization. The Mx3000E Pro software was used to calculate the threshold cycle. Relative gene expression was normalized to *cyclophillin A* expression using the ΔΔCt method [300].

3.4.4 Total Serum Chemerin Measurements

Serum chemerin levels were measured using a mouse chemerin enzyme-linked immunosorbent assay, as per manufacturer's instructions (Millipore, Billerica, Massachusetts). Briefly, sample wells were washed with wash buffer prior to the addition of assay buffer, quality controls, serum samples, and standards ranging from 3.125 to 200 ng/mL. Following a 1.5 hr incubation, sample wells were washed and incubated with the detection antibody for 1 hr. The wells were again washed, and the enzyme solution was added and incubated for 30 min. Next, the wells were washed, and the substrate solution was added and incubated for 30 min. Stop solution was added immediately afterwards, and the absorbance was measured at 450 nm and 590 nm.

3.4.5 Western Blotting

Approximately 500 mg of adipose, skeletal muscle or liver tissue was homogenized in 1.5 mL of ice cold subcellular fractionation buffer (250 mM sucrose, 20 mM Hepes pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) and centrifuged at 10,000 x g for 10 min at 4 °C. The protein concentration of the clarified homogenate was quantitated using a Lowry assay and 40 μg

were separated on a 15 % SDS-polyacrylamide gel and subsequently transferred overnight (25 V) to a nitrocellulose membrane. After rinsing with PBS, the membranes were incubated in blocking solution (5 % non-fat skim milk dissolved in Tris-buffered saline with 0.1 % Tween (TBST)) for 1 h at room temperature. Following this, the membranes were incubated overnight at 4 ^{o}C with an anti-mouse chemerin antibody (AF2325; R&D systems, Minneapolis, Minnesota) diluted 1:500 in blocking solution. After washing 4 x 5 min in TBST, the membrane was further incubated with a horseradish peroxidase conjugated donkey anti-goat IgG secondary antibody (1:10 000 in blocking buffer) for 1 hr at room temperature. After washing 4 x 5 min in TBST, the immunoreactive chemerin protein (approx 16 kDa) was visualized using ECL-plus™ reagent (GE Healthcare, Piscataway, New Jersey) and a Storm 840 phosphor imager (GE Healthcare).

3.4.6 Quantification of Bioactive Chemerin Using the CMKLR1 "Tango" Bioassay

HTLA cells, kindly provided by Dr. Gilad Barnea [222], were maintained in DMEM supplemented with 10 % FBS, 0.1 % penicillin/streptomycin, 100 µg/ml streptomycin, 0.5 mg/mL G418, 5 µg/mL puromycin and 0.2 mg/mL hygromycin. For assays, the cells were seeded on 96-well plates at a density of 12,000 cells per well in plating media (same as maintenance media, but without selection agents). After 24 h, for each well: 25 ng CMKLR1-TL-tTA plasmid, 25 ng pCMV-β-galactosidase reference plasmid and 50 ng carrier plasmid pBSK were added to 10 µL of Opti-Mem reduced serum media. After the sequential addition of 0.1 µL polyethylenimine, incubation for 10 min at room temperature and the addition of 40 µL plating media, the entire volume of resulting transfection mix was added to each well. After 24 h, the transfection mix was

removed and replaced with serum samples diluted 1:10 in a total of 50 µL Opti-Mem. Following an additional 24 h incubation, the media was aspirated, the cells were washed once with 100 µL of PBS and incubated for 5 min with shaking (10 000 RPM) in 100 µL of reporter lysis buffer (RLT, Promega, Nepean, Ontario) followed by a rapid freeze/thaw cycle to lyse the cells. For the luciferase assay, 10 µL of lysate was transferred to a 96-well white luminometer microplate. Luciferase activity was measured using luciferase assay reagent (Promega, Nepean, Ontario) and a Luminoskan Ascent Luminometer (Thermo Fisher Scientific, Waltham, Massachusetts). For the β-galactosidase assay, 30 µL of lysate was transferred to a clear 96-well plate incubated with 30 µL of 2x β-galactosidase assay buffer (Promega , Nepean, Ontario) for 15 min at 37 °C. The reaction was stopped by the addition of 100 µL of 1 M Na_2CO_3 and the absorbance at 420 nM was measured. The luciferase and β-galactosidase measurements were corrected for the respective blanks and sample activity was expressed as the ratio of luciferase/β-galactosidase activity. (Promega , Nepean, Ontario). A standard curve was derived from activity measurements of serial dilutions (0.1 – 30 nM) of recombinant mouse chemerin prepared in Opti-Mem and treated identically to serum samples. Apparent serum chemerin concentrations were extrapolated from a standard curve generated by nonlinear regression and fitting to a one-site binding hyperbola using GraphPad Prism v4.00 (Graphpad Software, LaJolla, California).

3.4.7 Glucose Tolerance Test

Glucose tolerance tests were performed on $Lep^{ob/ob}$, $Lepr^{db/db}$ and C57BL/6 littermate controls at 12 weeks of age, and DIO mice at 24 weeks of age. Mice were weighed prior to the test, and following an 18 h overnight fast, were injected IP with filter

sterilized D-Glucose (BDH Inc., Toronto, Ontario) at 2 mg/g and either PBS, 4 ng/g or 40 ng/g of recombinant human chemerin. Blood samples were collected from the saphenous vein at 0, 15, 30, 45, 60, 90, and 120 min post-injection, and glucose concentrations were measured using a glucometer (Freestyle Freedom®).

3.4.8 Serum Insulin Measurements

Serum insulin levels were measured using a rat/mouse insulin enzyme-linked immunosorbent assay as per manufacturer's instructions (Millipore, Billerica, Massachusetts). Briefly, sample wells were washed prior to the addition of assay buffer, matrix solution, quality controls, serum samples, standards ranging from 0.2 to 10 ng/mL, and detection antibody. Following a 2 hr incubation, sample wells were washed and incubated with enzyme solution for 30 min. Next, the wells were washed, and the substrate solution was added and incubated for 15 min. Stop solution was added immediately afterwards, and the absorbance was measured at 450 nm and 590 nm.

3.4.9 *In Vivo* Tissue Glucose Uptake During a Glucose Tolerance Test

Glucose tolerance tests were performed on $Lepr^{db/db}$ and C57BL/6 littermate controls at 12 weeks of age. Mice were weighed prior to the test, and following an 18 h overnight fast, were injected IP with filter sterilized D-Glucose (BDH Inc., Toronto, Ontario) at 2 mg/g, 10 µCi of 2-[1,2-^{3}H(N)]-Deoxy-D-Glucose (2-DOG) (Perkin Elmer, Waltham, Massachusetts), and either PBS or 40 ng/g of recombinant human chemerin. Blood samples were collected from the saphenous vein at 0, 15, 30, 45, and 60 min post-injection, and glucose concentrations were measured using a glucometer (Freestyle Freedom®). At 60 min, mice were anaesthetized and liver, skeletal muscle

(gastrocnemius), and epididymal white adipose tissue samples were snap frozen in liquid nitrogen. To determine glucose-specific activity (GSA), plasma samples from 0, 15, 30, 45, and 60 min were deproteinized using perchloric acid, and neutralized with $KHCO_3$. Radioactivity was measured using a scintillation counter, and GSA was calculated by determining the area under the curve of sample radioactivity divided by glucose concentration for the duration of the experiment. To determine tissue accumulation of 2-DOG, 100-500 mg of tissue was homogenized in distilled water, and the homogenate was transferred to perchloric acid. The sample was centrifuged to remove precipitated protein, and the supernatant was neutralized with $KHCO_3$. The precipitate was removed by centrifugation, and the radioactivity in the supernatant was measured in a scintillation counter. To calculate 2-DOG uptake, tissue radioactivity was divided by the glucose specific activity and the mass of the tissue homogenized.

3.4.10 Statistics

All data are expressed as mean ± SEM. All comparisons were performed using an unpaired t test, or a one or two-way analysis of variance (ANOVA), unless otherwise stated. A Bonferroni's test was used for *post-hoc* analysis of the significant ANOVA. A difference in mean values between groups was considered to be significant when $P < 0.05$.

3.5 Results

3.5.1 Characterization of the mRNA Levels of Chemerin and its Cognate Receptors

Previous studies have reported that the mRNA levels of *chemerin* and *CMKLR1* are highest in liver and white adipose tissue [228]. However, a relative comparison of the

mRNA levels of chemerin and its cognate receptors in white C57BL/6 adipose, liver, and skeletal muscle tissue, tissues with roles important in glucose homeostasis, has not been performed. Using quantitative real-time PCR, we found that mouse *chemerin* mRNA levels were significantly lower in skeletal muscle compared to white adipose and liver tissue (Figure 6). *CMKLR1* mRNA levels were approximately 5-fold lower in skeletal muscle and 36-fold lower in liver tissue relative to white adipose tissue. In contrast, the mRNA levels of *CCRL2* and *GPR1* were significantly higher in skeletal muscle tissue when compared to liver and white adipose tissue. To determine the effect of obesity and diabetes on the mRNA levels of *chemerin* and its receptors, leptin deficient (ob/ob) and leptin receptor deficient (db/db), mouse models were used. In ob/ob mice, *CMKLR1* mRNA was 2.3-fold lower in white adipose tissue, and 4.8-fold higher in skeletal muscle compared to congenic C57BL/6 controls (Figure 7). *Chemerin* mRNA levels were also significantly higher in ob/ob skeletal muscle compared to C57BL/6 controls (Figure 7). Similar to ob/ob mice, *CMKLR1* levels were significantly lower (2.7-fold) in white adipose tissue, and higher (4.3-fold) in skeletal muscle of db/db mice compared to C57BL/6 mice (Figure 8). In contrast to ob/ob mice, *CCRL2* mRNA levels were significantly higher in db/db white adipose tissue and lower in db/db liver. Also in contrast to ob/ob mice, *chemerin* mRNA was significantly higher in db/db liver tissue (Figure 8).

3.5.2 Quantitation of Serum Chemerin

Several recent studies have demonstrated that serum chemerin levels in humans are positively associated with characteristics of the metabolic syndrome, including obesity, plasma triacylglycerides, and blood pressure [229, 271, 272]. To determine if

Figure 6. Chemerin and Cognate Receptors are Differentially Expressed in Tissues Important in Glucose Homeostasis. Relative mRNA levels of *chemerin*, *CMKLR1*, *CCRL2*, and *GPR1* determined in C57BL/6 mouse white adipose, skeletal muscle, and liver tissues by quantitative real time PCR. White adipose served as the reference tissue (expression = 1.0) to which all other tissues were compared. N = 4-5. Each bar represents the mean ± SEM. *P<0.05, one-way ANOVA followed by Bonferroni's multiple comparison test.

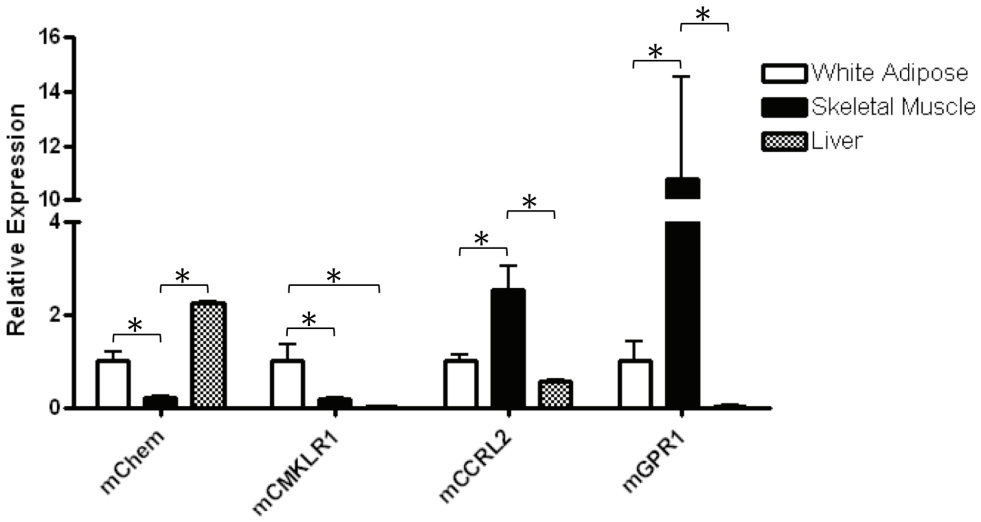

Relative Expression
16
14
12
10
4
2
0
White Adipose
Skeletal Muscle
Liver
mChem
mCMKLR1
mCCRL2
mGPR1
*

Figure 7. The mRNA Levels of Chemerin and its Cognate Receptors are Altered in ob/ob Mice. Relative mRNA levels of *chemerin*, *CMKLR1*, *CCRL2*, and *GPR1* determined in C57BL/6 and ob/ob white adipose, skeletal muscle, and liver tissue by quantitative real time PCR. C57BL/6 expression served as the reference (expression = 1.0) to which ob/ob mice were compared. N = 5-10. Each bar represents the mean ± SEM. *P<0.05, unpaired t test.

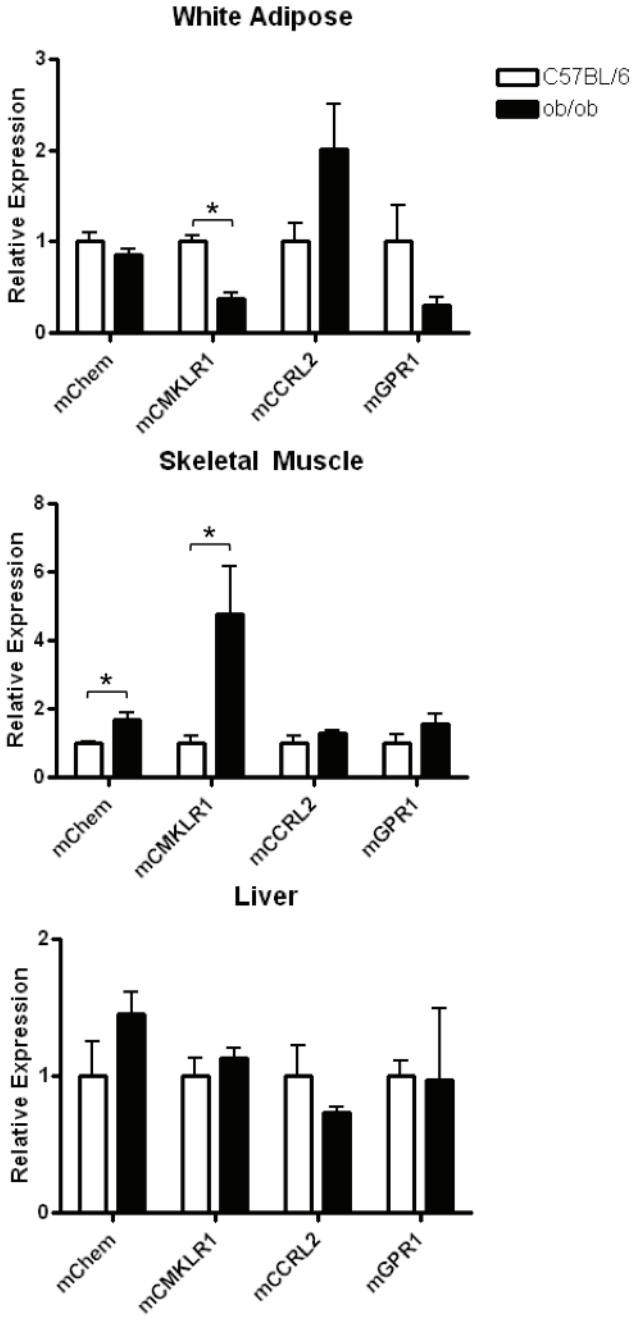
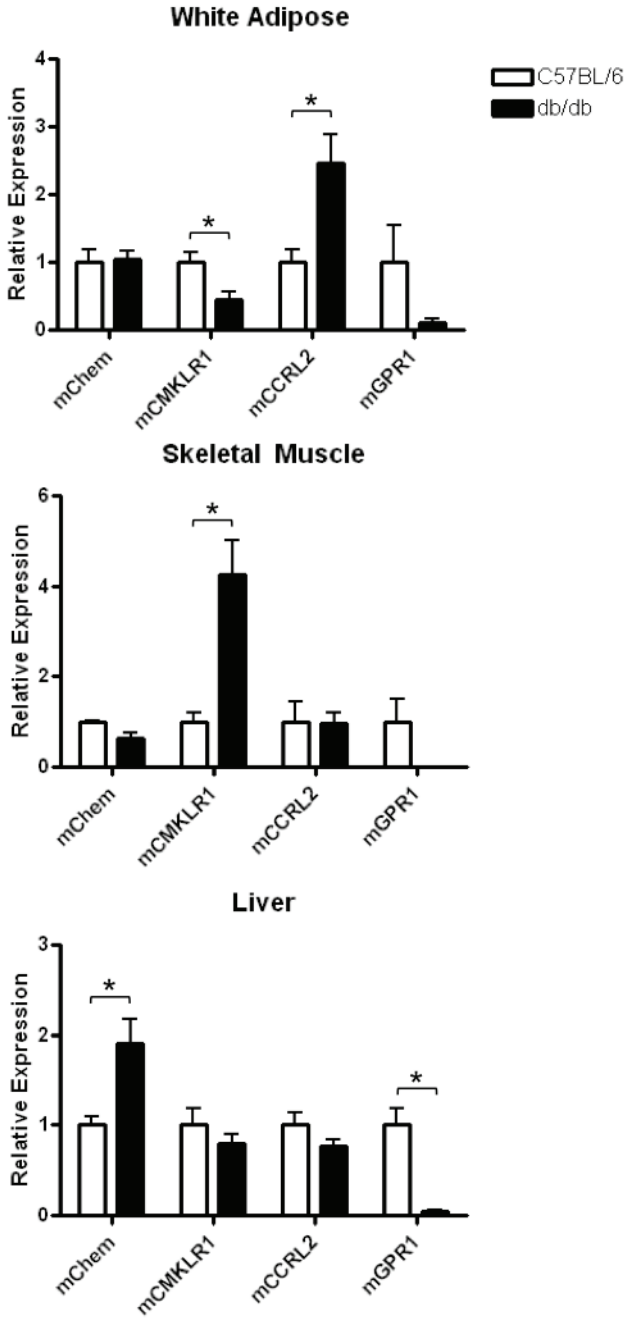
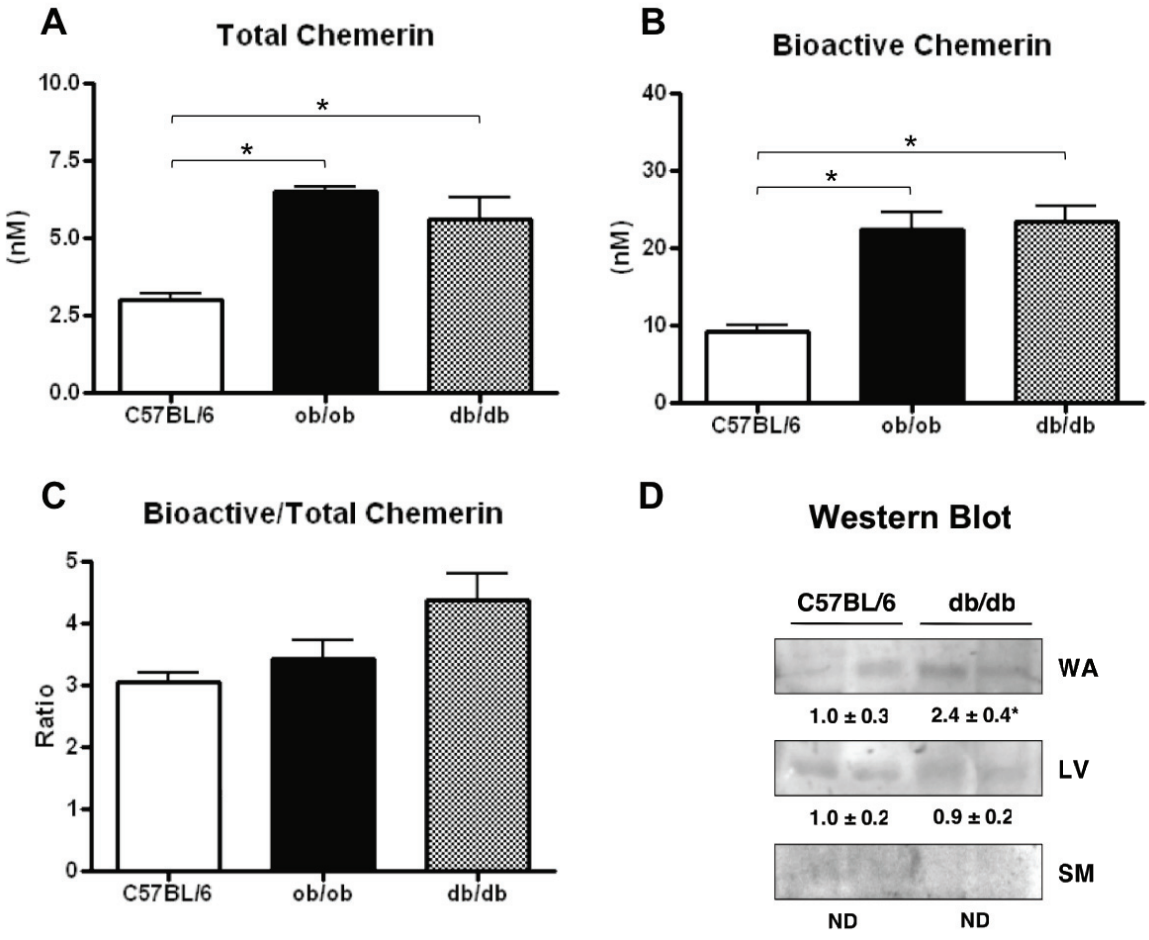

White Adipose
C57BL/6
ob/ob
Relative Expression
0
1
2
3
*
mChem
mCMKLR1
mCCRL2
mGPR1
Skeletal Muscle
Relative Expression
0
2
4
6
8
*
*
mChem
mCMKLR1
mCCRL2
mGPR1
Liver
Relative Expression
0
1
2
mChem
mCMKLR1
mCCRL2
mGPR1

Figure 8. The mRNA Levels of Chemerin and its Cognate Receptors are Altered in db/db Mice. Relative mRNA levels of *chemerin*, *CMKLR1*, *CCRL2*, and *GPR1* determined in C57BL/6 and db/db white adipose, skeletal muscle, and liver tissue by quantitative real time PCR. C57BL/6 expression served as the reference (expression = 1.0) to which ob/ob mice were compared. N = 5-10. Each bar represents the mean ± SEM. *P<0.05, unpaired t test.


White Adipose
C57BL/6
db/db
Relative Expression
0
1
2
3
4
*
*
mChem
mCMKLR1
mCCRL2
mGPR1
Skeletal Muscle
Relative Expression
0
2
4
6
*
mChem
mCMKLR1
mCCRL2
mGPR1
Liver
Relative Expression
0
1
2
3
*
*
mChem
mCMKLR1
mCCRL2
mGPR1

mouse serum chemerin levels also correlated with obesity, total chemerin levels were quantified in serum from C57BL/6, ob/ob, and db/db mice. In both ob/ob and db/db mice, total serum chemerin levels were approximately 2-fold higher than C57BL/6 controls (Figure 9A). Chemerin is secreted as an 18 kDa inactive proprotein, known as prochemerin, that can be rapidly converted into its active 16 kDa form by the proteolytic removal of the *C*-terminal amino acids by plasmin, carboxypeptidases or serine proteases of the coagulation, fibrinolytic and inflammatory cascades [219, 225, 232, 233, 236]. Consequently, it was important to determine if these elevated total serum chemerin levels corresponded to an increase in bioactive chemerin. A limitation of the ELISA is that it detects both prochemerin and bioactive chemerin (i.e. total chemerin). Using a CMKLR1-Tango assay [222], we detected a 2-fold greater level of bioactive chemerin in the serum of ob/ob or db/db mice compared to C57BL/6 controls (Figure 9B). Interestingly, the serum concentration of bioactive chemerin in all mice was 3-3.5-fold greater than the concentration of total chemerin (Figure 9C). To begin to elucidate the tissue source of the elevated serum chemerin with obesity/diabetes, total chemerin protein levels were analyzed by western blotting of white adipose, liver or skeletal muscle homogenates. Despite the high levels of mRNA for chemerin in adipose and liver, absolute chemerin immunoreactivity in these tissues was quite weak (Figure 9D). Total chemerin protein levels were 2.4-fold higher in adipose tissue homogenates prepared from obese, diabetic db/db mice compared to normo-glycemic C57BL/6 mice. In contrast, chemerin protein levels were similar in liver tissue homogenates prepared from either group of mice. In skeletal muscle, chemerin protein was undetectable by western blotting.

Figure 9. Serum Chemerin Levels are Elevated in Mouse Models of Obesity and Diabetes. Blood was collected using cardiac puncture, and was allowed to coagulate for 2 h. The resulting serum was analyzed for (A) total chemerin using a mouse chemerin ELISA, and (B) bioactive chemerin levels using a CMKLR1 Tango assay. (C) We then calculated the ratio of bioactive to total chemerin levels. N = 5. Each bar is the mean ± SEM. **P<0.01 vs. C57BL/6, one-way ANOVA followed by Bonferroni's multiple comparison test. (D) Total chemerin protein was also examined in white adipose (WA), liver (LV), and skeletal muscle (SM) tissues using western blotting. ND = not detected. Values represent mean relative densitometry data ± SEM. *P<0.05, unpaired t test.


A
Total Chemerin
10.0
7.5
5.0
2.5
0.0
(nM)
*
*
C57BL/6
ob/ob
db/db
B
Bioactive Chemerin
40
30
20
10
0
(nM)
*
*
C57BL/6
ob/ob
db/db
C
Bioactive/Total Chemerin
5
4
3
2
1
0
Ratio
C57BL/6
ob/ob
db/db
D
Western Blot
C57BL/6
db/db
WA
1.0 ± 0.3
2.4 ± 0.4*
LV
1.0 ± 0.2
0.9 ± 0.2
SM
ND
ND

3.5.3 The Impact of Chemerin on Blood Glucose Levels

Adipokines, including leptin, adiponectin, visfatin, resistin, omentin, and interleukin-6, modulate energy metabolism, insulin sensitivity, and glucose tolerance. Knockdown of *chemerin* expression by adenovirus-delivered shRNA in mature adipocytes causes a decrease in the expression genes important in glucose homeostasis and the pathogenesis of insulin resistance, including GLUT4, adiponectin, and leptin [228]. However, the systemic effects of chemerin on glucose homeostasis remain unknown. To investigate chemerin function *in vivo*, intraperitoneal injections of PBS, recombinant human chemerin, and human insulin were performed in 12 week old C57BL/6 mice, and blood glucose levels were monitored over a 2 h period. As expected, insulin significantly decreased blood glucose levels relative to vehicle (Figure 10). In contrast, intraperitoneal injections of recombinant human chemerin did not significantly impact blood glucose levels (Figure 10). Obesity and type II diabetes is associated with alterations in energy metabolism, glucose homeostasis, and resistance to the actions of insulin. Serum levels and tissue sensitivity of adipokines are also affected by the degree of adiposity and BMI. Therefore, glucose tolerance tests were performed in the presence and absence of chemerin with both normo-glycemic (C57BL/6) and diabetic (ob/ob, db/db and DIO) mouse models. Chemerin administration had no effect on glucose tolerance in C57BL/6 control mice (Figure 11A, 12A, 13A). In ob/ob mice, 40 ng/g of recombinant human chemerin exacerbated glucose intolerance, with significantly elevated blood glucose levels at 30 and 45 min (Figure 11B). The lower dose of 4 ng/g had no significant effect. Similar to the ob/ob results, chemerin exacerbated glucose intolerance in db/db mice, with significantly elevated blood glucose levels at 60 and 90 min (Figure 12B). Again, the lower dose of 4 ng/g did not have a significant effect.

Figure 10. Chemerin Treatment Does Not Affect Blood Glucose Levels in Wildtype Mice. C57BL/6 mice were fasted for 6 h, and then injected i.p with vehicle, chemerin (4 ng/g or 40 ng/g), or insulin (31.25 ng/g). Blood samples were collected over a 2 h period, and glucose levels were quantified. N = 5. Each bar is the mean ± SEM. P*<0.05 vs vehicle, two-way ANOVA followed by Bonferroni's multiple comparison test. Each bar represents mean ± SEM.

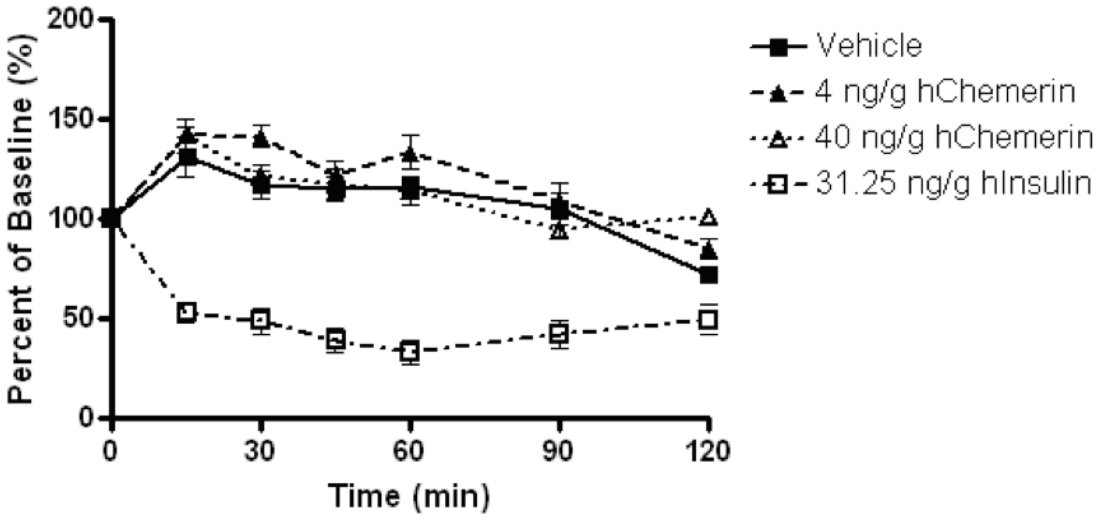

Percent of Baseline (%)
200
150
100
50
0
0
30
60
90
120
Time (min)
Vehicle
4 ng/g hChemerin
40 ng/g hChemerin
31.25 ng/g hInsulin

Figure 11. Chemerin Treatment Exacerbates Glucose Intolerance in ob/ob Mice. Vehicle or chemerin (4 ng/g or 40 ng/g) was injected i.p. to (A,C) C57BL/6 and (B,D) ob/ob mice with glucose (2 mg/g). Blood samples were collected over a 2 h period, and blood glucose levels were analyzed (A,B). Serum samples were also collected throughout the glucose tolerance test and analyzed for serum insulin levels (C,D). N = 10. Each bar represents mean ± SEM. P*<0.05, P**<0.01 vs. vehicle, two-way ANOVA followed by Bonferroni's multiple comparison test.

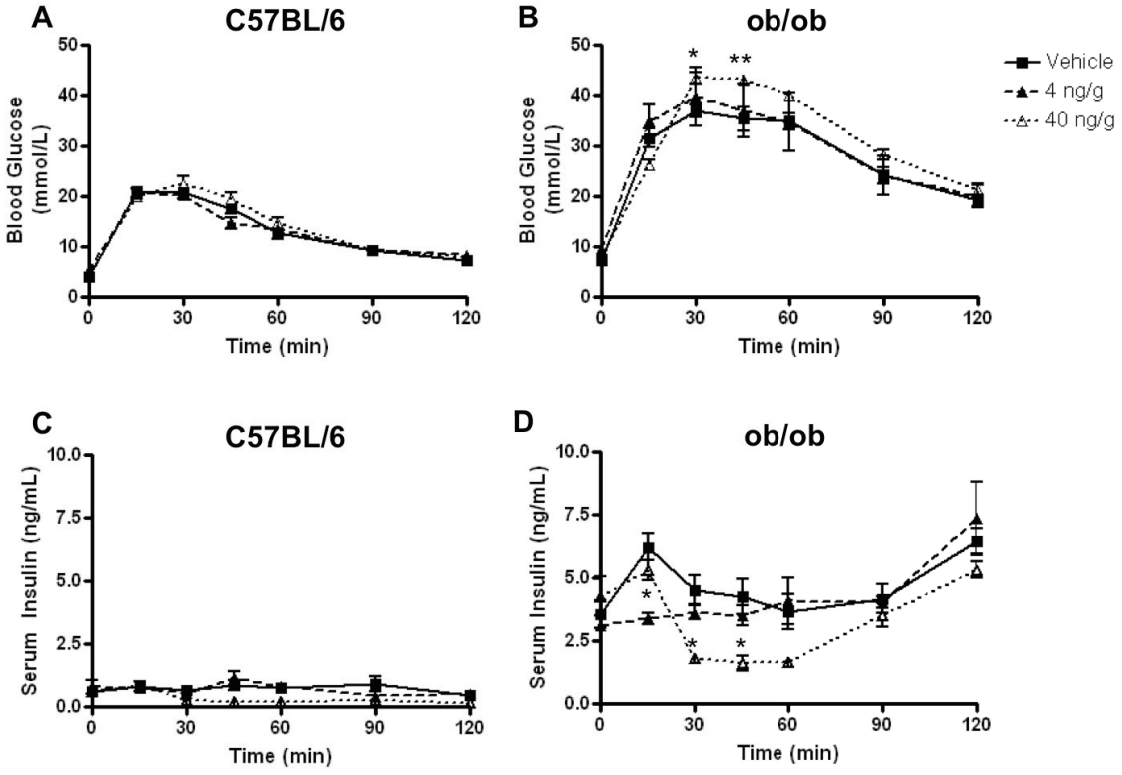

A
C57BL/6
Blood Glucose (mmol/L)
Time (min)
B
ob/ob
Blood Glucose (mmol/L)
Time (min)
Vehicle
4 ng/g
40 ng/g
C
C57BL/6
Serum Insulin (ng/mL)
Time (min)
D
ob/ob
Serum Insulin (ng/mL)
Time (min)

Figure 12. Chemerin Treatment Exacerbates Glucose Intolerance in db/db Mice. Vehicle or chemerin (4 ng/g or 40 ng/g) was injected i.p. to (A,C) C57BL/6 and (B,D) db/db mice with glucose (2 mg/g). Blood samples were collected over a 2 h period, and blood glucose levels were analyzed (A,B). Serum samples were also collected throughout the glucose tolerance test, and were analyzed for serum insulin levels (C,D). N = 10. Each bar represents mean ± SEM. P*<0.05, P**<0.01 vs. vehicle, two-way ANOVA followed by Bonferroni's multiple comparison test.

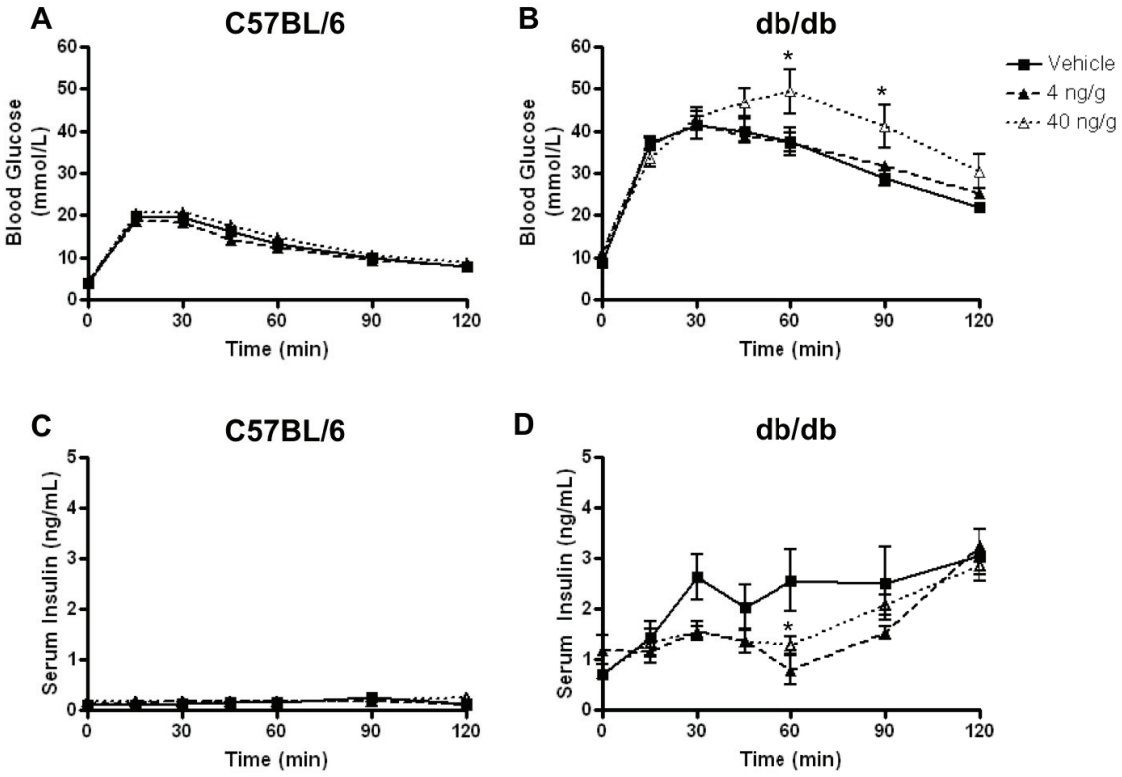
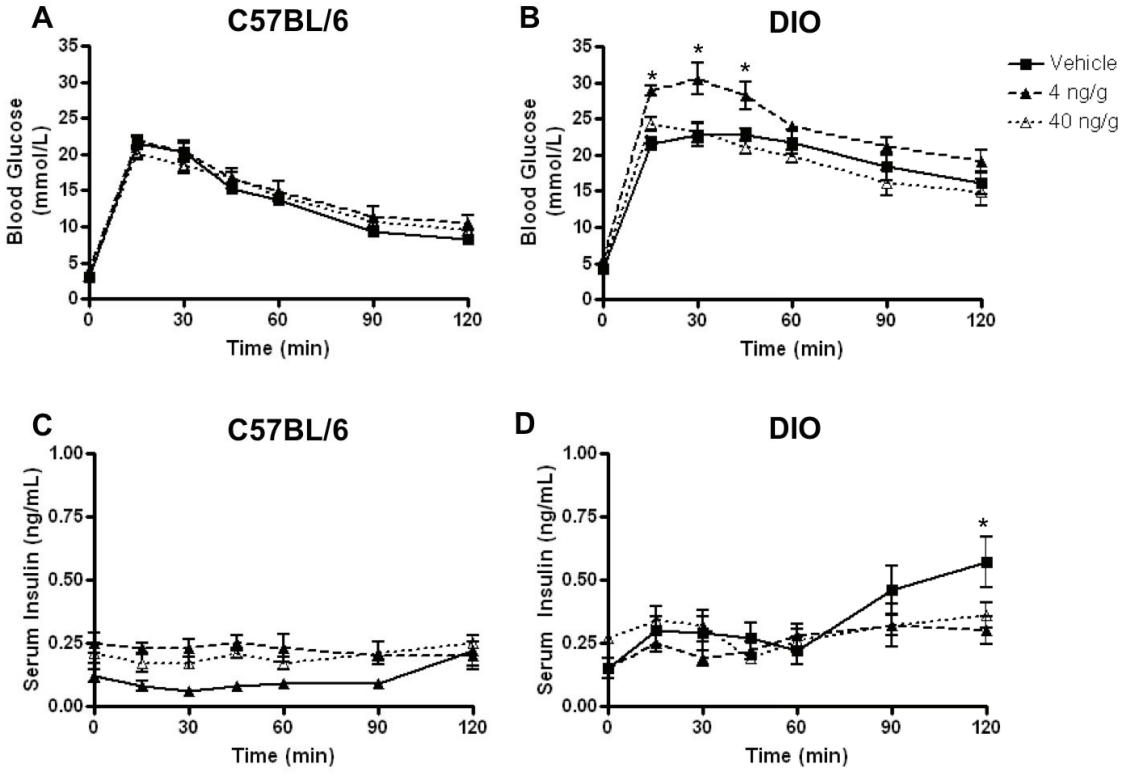

A
C57BL/6
Blood Glucose (mmol/L)
Time (min)
B
db/db
Blood Glucose (mmol/L)
Time (min)
Vehicle
4 ng/g
40 ng/g
C
C57BL/6
Serum Insulin (ng/mL)
Time (min)
D
db/db
Serum Insulin (ng/mL)
Time (min)

Figure 13. Chemerin Treatment Exacerbates Glucose Intolerance in DIO Mice. Vehicle or chemerin (4 ng/g or 40 ng/g) was injected i.p. to (A,C) C57BL/6 and (B,D) DIO mice with glucose (2 mg/g). Blood samples were collected over a 2 h period, and blood glucose levels were analyzed (A,B). Serum samples were also collected throughout the glucose tolerance test, and were analyzed for serum insulin levels (C,D). N = 10. Each bar represents mean ± SEM. P*<0.05 vs. vehicle, two-way ANOVA followed by Bonferroni's multiple comparison test.

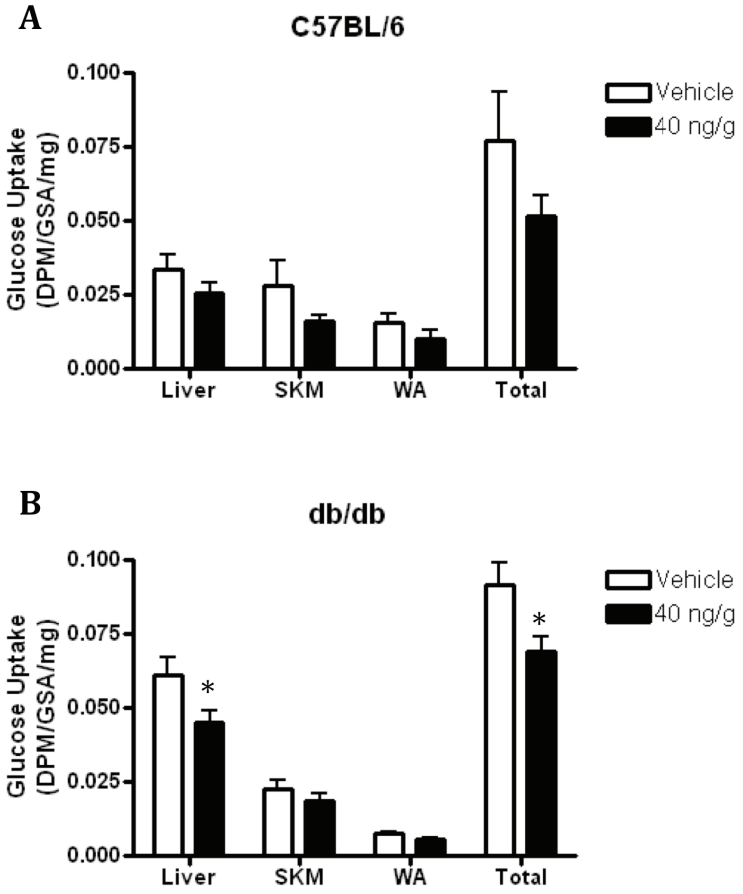

A
C57BL/6
Blood Glucose (mmol/L)
Time (min)
B
DIO
Vehicle
4 ng/g
40 ng/g
C
C57BL/6
Serum Insulin (ng/mL)
Time (min)
D
DIO
Serum Insulin (ng/mL)
Time (min)

Interestingly, 4 ng/g recombinant human chemerin exacerbated glucose intolerance in DIO mice, with significantly elevated blood glucose levels at 15, 30, and 45 min (Figure 13B). However, 40 ng/g did not have a significant effect on glucose tolerance in this model. To determine if the exacerbated glucose intolerance caused by chemerin administration to the obese/diabetic mouse models was associated with changes in serum insulin, levels of this hormone were measured throughout the GTT. In ob/ob mice, 40 ng/g of chemerin significant decreased serum insulin levels at 15, 30, and 45 min (Figure 11D). Similarly, 40 ng/g chemerin significantly reduced serum insulin levels in db/db mice at 60 min (Figure 12D). In DIO mice, both 4 ng/g and 40 ng/g chemerin significant decreased serum insulin levels at 120 min (Figure 13D). Consistent with the GTT data, chemerin treatment had no effect on serum insulin levels in C57BL/6 control mice (Figure 11C, 12C, 13C).

3.5.4 The Impact of Chemerin on *In Vivo* Tissue Glucose Uptake

To determine if the exacerbated glucose intolerance caused by chemerin *in vivo* (Figure 11B, 12B, 13B) was associated with a reduction in basal and/or insulin-mediated glucose uptake, we performed *in vivo* tissue glucose uptake experiments in db/db mice. As the maximum effect of 40 ng/g chemerin was seen at 60 min in the GTT (Figure 12B), this endpoint was selected for the glucose uptake assay. Consistent with GTT results, chemerin had no significant impact on tissue glucose uptake in C57BL/6 control mice (Figure 14A). However, chemerin treatment significantly decreased both liver and total tissue (white adipose, liver and skeletal muscle) glucose uptake (Figure 14B). A highly reproducible decrease in white adipose tissue glucose uptake was also observed with

Figure 14. Chemerin Decreases *In Vivo* Tissue Glucose Uptake in db/db Mice. Vehicle or chemerin (4 ng/g or 40 ng/g) was injected i.p. to (A) C57BL/6 and (B) db/db mice with glucose (2 mg/g) and 10 μCi of 2-[1,2-^{3}H(N)]-Deoxy-D-Glucose. Blood samples were collected over a 1 h period, and blood glucose concentration and radioactivity were measured. After 60 min, mice were euthanized and tissues were snap frozen, homogenized, and radioactivity measured. Glucose specific activity (GSA) was calculated by dividing blood radioactivity by blood glucose concentration and calculating the area under the curve. Tissue radioactivity (DPM) was normalized GSA and the mass of tissue homogenized. N = 5-10. Each bar represents the mean ± SEM. *P<0.05, unpaired t test.


A
C57BL/6
Glucose Uptake (DPM/GSA/mg)
0.100
0.075
0.050
0.025
0.000
Liver
SKM
WA
Total
Vehicle
40 ng/g
B
db/db
Glucose Uptake (DPM/GSA/mg)
0.100
0.075
0.050
0.025
0.000
*
*
Liver
SKM
WA
Total
Vehicle
40 ng/g

chemerin treatment of db/db mice, however this effect just failed to achieve statistical significance (P=0.06).

3.6 Discussion

Herein, we report for the first time that the novel adipokine chemerin exacerbates glucose intolerance in mouse models of obesity and diabetes. Consistent with recent human data [229, 271, 272], we observed a significantly higher amount of serum chemerin in two different genetic mouse models of obesity. Furthermore, our novel findings are the first to evaluate the mRNA levels of *chemerin* and its cognate receptors in white adipose, liver, and skeletal muscle tissue of mouse models of obesity.

In mice, *chemerin* and *CMKLR1* mRNA levels are highest in liver and adipose tissue [219, 228, 283]. However, these studies have not reported the mRNA levels of the novel chemerin receptors *CCRL2* and *GPR1*. The recently identified chemerin receptor CCRL2 is believed to be an atypical silent or nonsignalling receptor, that binds chemerin and increases local concentrations of the bioactive peptide [221]. Another recent study reported that GPR1, a GPCR closely related to CMKLR1, is also activated by chemerin, with an EC_{50}, of 1 nM, compared to 24 nM for CMKLR1 [222]. Consistent with previous studies, we observed that *chemerin* mRNA levels were highest in white adipose and liver tissue, and *CMKLR1* levels were highest in white adipose tissue. For the first time, we report here that the mRNA levels of *CCRL2* and *GPR1* are significantly higher in skeletal muscle tissue, when compared to both white adipose and liver tissue. This suggests that under normal conditions, the relative importance of GPR1 mediated chemerin signaling in skeletal muscle would be greater than CMKLR1. Previously we reported that *CMKLR1* mRNA levels were modestly, but not significantly lower in visceral white adipose tissue

of ob/ob mice versus C57BL6/J controls [228]. In the present study, we observed a significant decrease in mRNA levels of *CMKLR1* in white adipose tissue, and a significant increase in skeletal muscle tissue of ob/ob and db/db mice compared to C57BL/6 controls. The reason for this discrepancy may be that for the present study we used 12-13 week old mice, which have a more severe obese/diabetic phenotype compared to the younger mice used for the previous study. The role of chemerin in skeletal muscle energy metabolism and the effect of *CMKLR1* overexpression *in vitro* has not yet been examined, but a decrease in *CMKLR1* mRNA levels in white adipose tissue and an increase in skeletal muscle tissue suggests a redistribution in the targeting of chemerin activity mediated through this receptor. *GPR1* mRNA levels showed a trend to decrease in ob/ob white adipose, and db/db white adipose, skeletal muscle, and were significantly decreased in db/db liver tissue. However, the functional role and signaling cascade of GPR1 remains unknown, and further studies are required to determine the effect of a reduction in *GPR1* mRNA levels. The mRNA levels of *CCRL2* were significantly increased in white adipose tissue of db/db mice, and trended towards an increase in ob/ob white adipose tissue. Interestingly, chemerin was initially described as a chemoattractant protein with a role in adaptive and innate immunity [218-220, 226, 230, 232, 236]. Therefore, by increasing the local concentration of bioactive chemerin, an elevation in *CCRL2* mRNA levels may contribute to the increase in leukocyte infiltration observed in white adipose tissue found in obesity.

Many studies have demonstrated that serum levels of adipokines, including leptin and adiponectin are affected by the degree of adiposity and BMI [16, 288-290, 295-299]. Consistent with recent human studies [229, 272], we found that total serum chemerin

levels were elevated in mouse models of obesity and diabetes. The elevation of chemerin mRNA levels in skeletal muscle of ob/ob mice, and liver tissue of db/db mice is a possible explanation for the elevated serum chemerin levels in these mice. However, despite the 2-fold higher levels of chemerin mRNA levels in db/db when compared to C57BL/6 mice, total chemerin protein levels in liver were not significantly different between these models. Furthermore, chemerin protein was undetectable in skeletal muscle homogenates prepared from C57BL/6 or db/db mice. However, total chemerin protein levels were elevated 2.4-fold in white adipose homogenates prepared from db/db versus C57BL/6 mice, suggesting that white adipose-derived chemerin contributes to the elevated circulating chemerin levels. Since the mRNA and protein levels do not coincide, we can not definitely conclude that white adipose tissue is the source of elevated serum chemerin levels. However, this result is consistent with data derived from adipose tissue explants obtained from obese patients which are characterized by significantly higher chemerin secretion than lean control subjects [278]. Given that white adipose chemerin mRNA levels were not correspondingly higher, it is most likely that the elevated adipose, and possibly, serum chemerin derives from changes in post-translational processes (i.e. proteolytic processing, secretion).

Chemerin is secreted as an 18 kDa inactive proprotein that is processed into its active 16 kDa form which is responsible for receptor binding and physiological activity [219, 220, 226, 230, 232]. However, the serum levels of bioactive chemerin, or the effect of obesity on these levels have not previously been reported in the literature. This is significant as a limitation of the chemerin ELISA is that it detects both prochemerin and bioactive chemerin. Using the Tango assay described here, we determined that serum

total chemerin protein levels and bioactive chemerin levels were approximately 2-fold higher in mouse models of obesity compared to C57BL/6 mice. However, the concentration of bioactive chemerin levels in serum was 3-fold higher than serum levels of total chemerin protein. Both the ELISA and the Tango assay used the same recombinant mouse chemerin to obtain a standard curve. However, the Tango assay standard curve is measuring the activation of CMKLR1 by a single form of chemerin, but studies have shown that several chemerin derivatives have different levels of activity. Several peptides between 8-19 amino acids in length that correspond to the c-terminus of chemerin have similar activity to recombinant mouse chemerin [236, 237]. Another study suggests that chemerin activation requires two cleavages, with the first producing bioactive chemerin with very low activity, and the second producing fully activated bioactive chemerin [233]. Therefore, serum levels of bioactive chemerin are likely higher than the concentration of total chemerin protein because there are chemerin derivatives in the serum with activities similar to or greater than recombinant mouse chemerin used to generate the standard curve. Thus, we believe it is critical to measure both levels as disease and/or drugs have the potential to affect chemerin expression, secretion, and processing.

Studies have shown that both serum levels and the physiological response to many adipokines are altered with obesity. Consistent with this, recombinant chemerin administration exacerbated glucose intolerance in obese/diabetic (ob/ob, db/db and DIO), but not normo-glycemic C57BL/6 mouse models. The consistent impact of chemerin on glucose tolerance in all of the models of obesity/diabetes suggests that leptin or leptin receptor deficiency is not directly responsible for the observed differences in glucose

tolerance. However, this cannot be ruled out based upon the present data as altered leptin signaling (i.e. leptin resistance) is common with obesity. Because chemerin only affected glucose intolerance in obese/diabetic mice, the most likely mechanisms are by reducing serum insulin levels, or by further modulation of the already perturbed insulin signaling cascade. By collecting serum throughout the GTT and quantifying serum insulin levels, we determined that chemerin treatment significantly reduced insulin levels in obese and diabetic mice, but had no effect in C57BL/6 mice. Consistent with the glucose tolerance data, total tissue glucose uptake significantly decreased in obese mice treated with chemerin, but not in C57BL/6 mice. Glucose uptake was also consistently reduced in white adipose tissue, however statistical significance was not attained ($p=0.06$) in this set of experiments. No effect of chemerin was apparent for skeletal muscle glucose uptake in normo-glycemic or obese/diabetic mice. Given that the greatest impact of chemerin was on hepatic glucose uptake, modulation of GLUT4 is unlikely as a mechanism as this transporter does not contribute substantially to insulin stimulated glucose uptake in this tissue. Rather, following an increase in blood glucose concentration, glucose uptake into liver tissue is principally facilitated by GLUT2, a passive glucose transporter [301]. Glucose is then phosphorylated by glucokinase, and used in various metabolic pathways, such as glycogen synthesis. GLUT2 is also highly expressed in pancreatic beta cells, and initiates insulin secretion by increasing the concentration of glucose within the cell. Since we observed both a decrease in serum insulin levels, and a decrease in liver glucose uptake, it is possible that chemerin is reducing GLUT2 mediated glucose transport in these tissues, thus resulting in a decrease in glucose uptake and insulin secretion. In addition, studies have shown that CMKLR1 activation results in intracellular calcium

release, and phosphorylation of ERK kinases [230]. This is interesting because the insulin secretion cascade also causes intracellular calcium relase and ERK activation, suggesting that chemerin treatment may impair insulin secretion by competing for these responses. Further studies are required to determine if any direct interaction between chemerin signaling, GLUT2 mediated glucose uptake, and insulin secretion exist.

In summary, we provide evidence that serum chemerin levels are elevated in obesity and diabetes, and that chemerin exacerbates glucose intolerance in these models by decreasing serum insulin levels and glucose uptake in liver tissue. Thus, further characterization of the function of chemerin and CMKLR1 and GPR1 signaling in hepatocytes and pancreatic β-cells has the potential to lead to novel therapeutic approaches for the treatment of obesity and type II diabetes.

Chapter IV: Targeted Disruption of the Chemokine Like Receptor-1 (CMKLR1) Gene Causes Reduced Adiposity And Glucose Intolerance

4.1 Manuscript Status and Student Contribution

The figures and text presented in this chapter have been reproduced with copyright permission (Appendix I) from the article:

Ernst, M.C., Haidl, I.D., Zúñiga, L.A., Dranse, H.J., Rourke, J.L., Zabel, B.A., Butcher, E.C. and Sinal, C.J. Targeted disruption of the chemokine like receptor-1 (CMKLR1) gene is associated with reduced adiposity and glucose intolerance. (Submitted to *Endocrinology* July 2011).

As first author on this article, I performed all of the experiments, analysis, data interpretation, and statistics with technical assistance from Helen. J. Dranse and Jillian L. Rourke. I wrote the manuscript with critical evaluation from Dr. Ian Haidl, Dr. Luis A. Zúñiga, Dr. Brian A. Zabel, Dr. Eugene C. Butcher, and Dr. Christopher Sinal.

4.2 Abstract

Adipose tissue secretes bioactive signaling molecules, termed adipokines, which regulate various biological functions including appetite, energy balance, glucose homeostasis, and inflammation. Chemerin is a novel adipokine that regulates adipocyte differentiation and metabolism by binding to and activating the G protein-coupled receptor (GPCR) chemokine like receptor-1 (CMKLR1). In the present study we investigated the impact of CMKLR1-deficiency on adipose development, glucose homeostasis and inflammation *in vivo*. Herein we report that regardless of diet (low or high fat), CMKLR1-/- mice had lower food consumption, total body mass, and percent body fat compared to wildtype controls. CMKLR1-/- mice also exhibited decreased hepatic and white adipose tissue TNFα and IL-6 mRNA levels coincident with decreased hepatic dendritic cell infiltration, decreased adipose CD3+ T cells and increased adipose natural killer cells. CMKLR1-/- mice were also glucose intolerant compared to wildtype mice, and this was associated with decreased glucose stimulated insulin secretion as well as decreased skeletal muscle and white adipose tissue glucose uptake. Collectively, these data provide compelling evidence that CMKLR1 influences adipose tissue development, inflammation, and glucose homeostasis, and may contribute to the metabolic derangements characteristic of obesity and obesity-related diseases.

4.3 Introduction

Obesity is characterized by an accumulation of excess white adipose tissue (WAT), and is associated with a variety of metabolic derangements that increase the risk for developing a number of diseases including hypertension, cardiovascular disease, and type II diabetes [11, 12]. Despite the clear link between obesity and the development of

these prevalent diseases, the mechanisms responsible for this relationship are not fully understood. In addition to serving an important role in energy storage, adipose tissue secretes a number of bioactive signaling molecules known as adipokines that affect many physiological processes, including appetite and energy balance, insulin sensitivity, and glucose uptake [13-15].

Chemerin is a recently discovered secreted protein with a role in adaptive and innate immunity [219, 225-227]. The first recognized function of chemerin, acting through CMKLR1, was to promote chemotaxis of dendritic cells and macrophages [219, 226]. Subsequently, chemerin and CMKLR1 were shown to be highly expressed in WAT [228, 229]. Chemerin expression and secretion increases dramatically with adipogenesis, and loss of chemerin or CMKLR1 in preadipocytes severely impairs adipocyte differentiation *in vitro* [228, 265]. Chemerin may also contribute to adipose tissue inflammation commonly found with obesity by serving as a chemoattractant for various types of immune cells [217]. Elevated serum chemerin levels reported in obese humans and rodents [229, 241, 242, 271, 272, 276, 279, 302-304] suggests that chemerin may contribute to the dysregulation of glucose metabolism that often occurs with obesity. In the present study, we examined the impact of genetic ablation of the chemerin receptor CMKLR1 on adiposity, inflammation and glucose metabolism in a mouse model of obesity. We report for the first time that loss of chemerin/CMKLR1 *in vivo* profoundly reduces adipose tissue accumulation, modifies white adipose immune cell infiltration and is associated with undesirable changes in insulin secretion and tissue glucose uptake.

4.4 Materials and Methods

4.4.1 Animal Protocol and Housing

All protocols and procedures were approved by the Dalhousie University Committee on Laboratory Animals and are in accordance with the Canadian Council on Animal Care guidelines. CMKLR1 knockout mice were originally generated by Deltagen and fully backcrossed into the C57BL/6 background. Mice were placed on a low fat (LF; 10 kcal% fat; D12450B; Research Diets, New Brunswick, NJ) or high fat (HF; 60 kcal% fat; D12492) beginning at 6 weeks of age. The fat source in the diets was derived from lard (LF 4 %, HF 54 %) and soyabean oil (LF 6 %, HF 6 %). For paired feeding, wildtype and CMKLR1 -/- mice were fed 2.2 g/day of HF diet for 6 weeks.

4.4.2 Dual Energy X-Ray Absorptiometry

Mice were anesthetized using isoflurane and whole body measurements of prostrate mice, excluding the head, were made by dual energy x-ray absorptiometry (DEXA; Lunar PIXImus2, GE Medical Systems). The DEXA instrument was calibrated before each use and one person performed all scans.

4.4.3 Blood Chemistry

Serum levels of insulin, leptin, adiponectin, chemerin, IL-6, and TNFα were determined using enzyme-linked immunosorbent assays, as per manufacturer's instructions (R&D systems, Minneapolis, MN; Millipore, Billerica, MA). Blood glucose levels were measured using a glucometer (Freestyle Freedom®, Abbott Laboratories, Saint-Laurent, Canada).

4.4.4 Histology

Liver samples were fixed in 10 % acetate buffered formalin for 48 h and subsequently embedded in paraffin. Deparaffinized sections (5 μM) were stained with Harris Hematoxylin and Eosin Y (Sigma Aldrich). Images were captured using a Zeiss Axiovert 200 inverted microscope with an attached Hamamatsu ORCA-R2 digital camera.

4.4.5 Quantitative Real-Time PCR

RNA isolation and quantitative real-time PCR was performed as described previously [279]. Primer sequences are shown in Table 2. Relative gene expression was normalized to *cyclophillin A* expression using the ΔΔCt method [300].

4.4.6 Flow Cytometry

Mice were perfused with PBS, and liver and WAT were collected. Tissues were minced using scissors to a homogeneous consistency in 2 ml HEPES buffer and incubated with 1000 U collagenase IV (liver) and 2500 U of collagenase I (WAT) respectively for 120 min at 37 ^{o}C in a shaking incubator at 200 rpm. The resulting suspension was passed through a 75 μm mesh filter to remove undigested tissue. The liver filtrate was centrifuged (30 x g, 3 min) and the supernatant was transferred and centrifuged (300 x g, 10 min). The resulting pellet was resuspended in 35% percoll and centrifuged (800 x g, 20 min). The remaining red blood cells were lysed by incubating with RBC lysis buffer (140 mM NH_4Cl, 20 mM Tris-Cl, pH 7.2) for 3 min, and the resulting cell suspension was centrifuged (800 x g, 5 min). The pellet was resuspended in FACS buffer (2.0 % FBS and 20 mM sodium azide in PBS). The WAT filtrate was centrifuged (50 x g, 5 min) and the

Table 2. Quantitative Real-Time PCR Primer Sequences.

Gene	Accession Number	Sequence (5' -> 3')
Cyclophillin A	NM_008907	Fw GAG CTG TTT GCA GAC AAA GTT C
		Rv CCC TGG CAC ATG AAT CCT GG
IL-6	NM_031168	Fw TAG TCC TTC CTA CCC CAA TTT CC
		Rv TTG GTC CTT AGC CAC TCC TTC
TNFα	NM_013693	Fw CCC TCA CAC TCA GAT CAT CTT CT
		Rv GCT ACG ACG TGG GCT ACA G

supernatant was transferred and centrifuged (200 x g, 10 min). The resulting pellet was resuspended in RBC lysis buffer, incubated for 3 min at room temperature, and the resulting cell suspension was centrifuged (200 x g, 5 min). The pellet was resuspended in FACS buffer. The cells were analyzed by flow cytometry for the expression of Ly-6G, F4/80, and CD11c to identify neutrophils as Ly-6G$^+$, F4/80$^-$, macrophages as Ly-6G$^-$, F4/80$^+$, and dendritic cells as F4/80$^-$, CD11c$^+$, and CD3, CD19, and NK1.1, to identify B cells as CD3$^-$, CD19$^+$, CD3+ T cells as CD3$^+$, NK1.1$^-$, natural killer T cells as CD3$^+$, NK1.1$^+$, and natural killer cells as CD3$^-$, NK1.1$^+$. Cells were collected on a FACSAria system (BD Biosciences) and were analyzed with FCS Express version 3.0 software (DeNovo Software, Los Angeles, CA).

4.4.7 Insulin Sensitivity and Glucose Tolerance Tests

Insulin sensitivity and glucose tolerance tests were performed as previously described [279].

4.4.8 Activity and Indirect Calorimetry

Wildtype and CMKLR1-/- mice fed a LF or HF diet for 6 weeks were individually housed in Panlab Physiocage 00 for 24 h for activity and indirect calorimetry analysis (Panlab, Holliston, MA, USA).

4.4.9 *In Vivo* Tissue Glucose Uptake During a Glucose Tolerance Test

Glucose tolerance tests were performed on LF and HF wildtype and CMKLR1-/- at week 6. Mice were weighed prior to the test, and following an 18 h overnight fast, were injected IP with filter sterilized D-Glucose (BDH Inc., Toronto, Canada) at 2 mg/g, and

10 μCi of 2-[1,2-^{3}H(N)]-Deoxy-D-Glucose (2-DOG) (Perkin Elmer, Waltham, MA). Blood samples were collected from the saphenous vein at 0, 15, 30, 45, and 60 min post-injection, and glucose concentrations were measured using a glucometer (Freestyle Freedom®, Abbott Laboratories, Saint-Laurent, Canada). At 60 min, mice were euthanized and liver, skeletal muscle (gastrocnemius), and WAT were snap frozen in liquid nitrogen. To determine glucose-specific activity (GSA), plasma samples from 0, 15, 30, 45, and 60 min were deproteinized using perchloric acid, and neutralized with $KHCO_3$. Radioactivity was measured using a scintillation counter, and GSA was calculated by determining the area under the curve of sample radioactivity divided by glucose concentration for the duration of the experiment. To determine tissue accumulation of 2-DOG, 100-500 mg of tissue was homogenized in distilled water, and the homogenate was transferred to perchloric acid. The sample was centrifuged to remove precipitated protein, and the supernatant was neutralized with $KHCO_3$. The precipitate was removed by centrifugation, and the radioactivity in the supernatant was measured in a scintillation counter. To calculate 2-DOG uptake, tissue radioactivity was divided by mass of tissue homogenized and the glucose specific activity.

4.4.10 Statistics

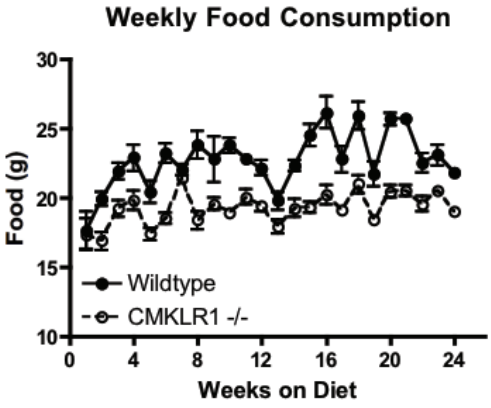
All data are expressed as mean ± SEM. All comparisons were performed using an unpaired t-test, or a one or two-way analysis of variance (ANOVA), unless otherwise stated. Bonferroni's test was used for *post-hoc* analysis of the significant ANOVA.

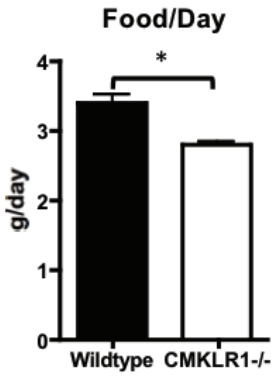
4.4 Results

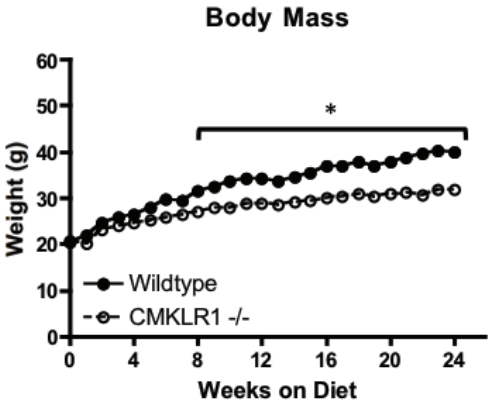
4.5.1 CMKLR1-Deficiency Reduces Food Consumption, Body Mass and Adiposity

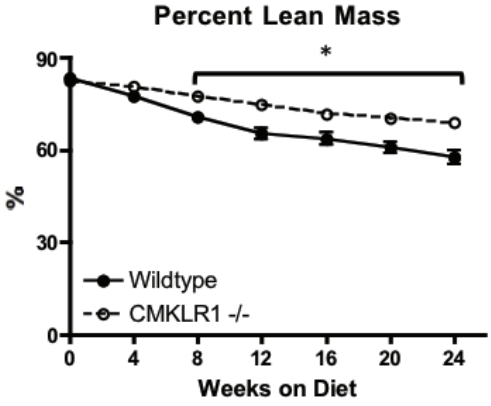
To determine the effect of CMKLR1 loss on food consumption and body weight, wildtype and CMKLR1-/- mice were fed a low fat (LF; 10 kcal% fat) or high fat (HF; 60 kcal% fat) diet for 24 weeks. Regardless of diet, the food consumption of CMKLR1-/- mice was lower at all measured time points and was approximately 25 % less than that of wildtype mice when expressed as daily food consumption (Figures 15, 16). A paired feeding study in which both wildtype and CMKLR1 -/- mice were limited to the daily food consumption of CMKLR1 -/- mice (2.2 g/day) demonstrated that reducing wildtype food intake to CMKLR1 -/- levels not only ablated differences in body weight, but two-way ANOVA analysis indicated that HF pair fed CMKLR1-/- mice had significantly greater body mass compared to wildtype mice, suggestive of a difference in metabolic rates between the genotypes (Figure 17). However, post-hoc analysis did not indentify any individual time points as being significantly different. CMKLR1-/- mice exhibited significantly lower body weights compared to wildtype mice beginning at week 8 (LF) or week 3 (HF) and these differences persisted for the remainder of the study (Figures 15, 16). DEXA analysis revealed a significantly higher lean mass for LF fed CMKLR1-/- mice from weeks 8 through 24 and for HF fed mice from weeks 4 through 12 (Figures 15, 16). However, the difference in total body mass was largely a consequence of differing fat mass as LF fed CMKLR1-/- mice exhibited significantly lower percent body fat from weeks 8 through 24, while HF fed CMKLR1-/- mice had significantly lower percent body fat from weeks 4 through 16 (Figures 15, 16). Activity levels and indirect calorimetry were also used as a measure of energy expenditure. Similar activity levels were observed

Figure 15. Low Fat Fed CMKLR1-/- Mice have Lower Food Consumption, Body Mass, and Percent Body Fat Compared to Wildtype Mice. Weekly and daily food consumption and total body mass of wildtype and CMKLR1-/- mice fed a LF diet for 24 weeks. Percent fat and lean mass were measured at week 0, 4, 8, 12, 16, 20, and 24 using DEXA analysis. Values are expressed as means ± SEM. *$p < 0.05$, comparing wildtype to CMKLR1-/- mice. N=6-10 mice/group.


Weekly Food Consumption
Food (g)
Weeks on Diet
Wildtype
CMKLR1 -/-


Food/Day
g/day
*
Wildtype
CMKLR1-/-


Body Mass
Weight (g)
*
Weeks on Diet
Wildtype
CMKLR1 -/-


Percent Lean Mass
%
*
Weeks on Diet
Wildtype
CMKLR1 -/-

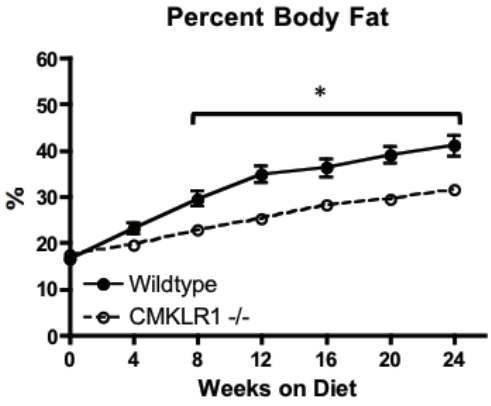
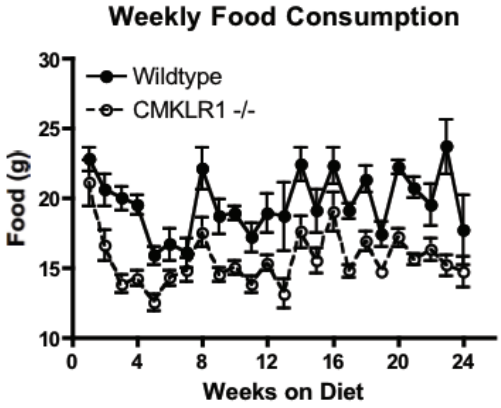
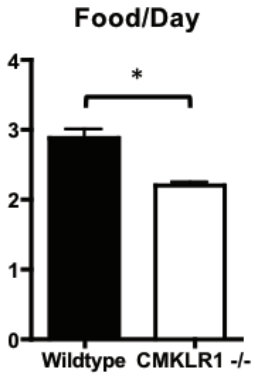
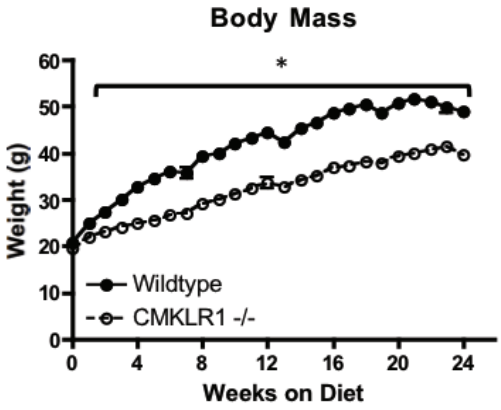
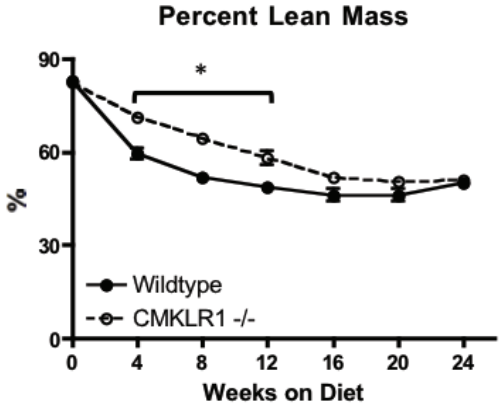

Percent Body Fat
%
*
Weeks on Diet
Wildtype
CMKLR1 -/-

Figure 16. High Fat Fed CMKLR1-/- Mice have Lower Food Consumption, Body Mass, and Percent Body Fat Compared to Wildtype Mice. Weekly and daily food consumption and total body mass of wildtype and CMKLR1-/- mice fed a HF diet for 24 weeks. Percent fat and lean mass were measured at week 0, 4, 8, 12, 16, 20, and 24 using DEXA analysis. Values are expressed as means ± SEM. *$p < 0.05$, comparing wildtype to CMKLR1-/- mice. N=6-10 mice/group.


Weekly Food Consumption
Wildtype
CMKLR1 -/-
Food (g)
Weeks on Diet


Food/Day
*
Wildtype
CMKLR1 -/-


Body Mass
*
Weight (g)
Wildtype
CMKLR1 -/-
Weeks on Diet


Percent Lean Mass
*
%
Wildtype
CMKLR1 -/-
Weeks on Diet

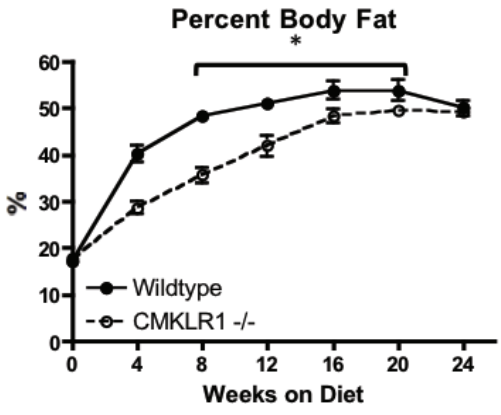
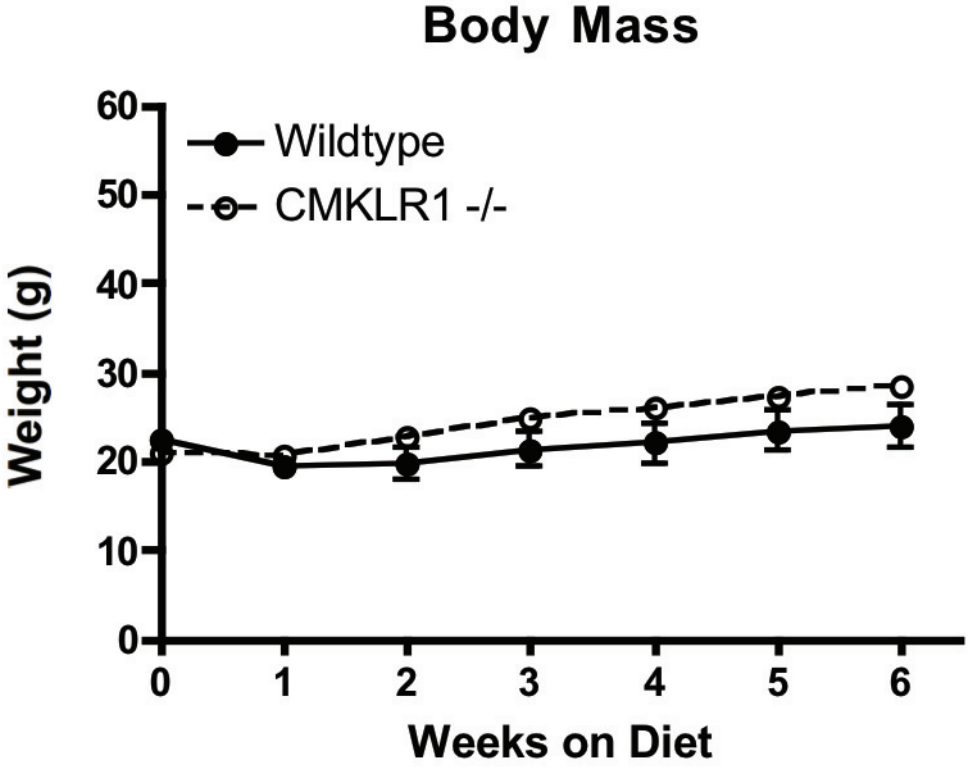

Percent Body Fat
*
%
Wildtype
CMKLR1 -/-
Weeks on Diet

Figure 17. Paired High Fat Fed CMKLR1 -/- Mice have a Higher Body Mass Than Wildtype Mice. Weekly total body mass of wildtype and CMKLR1 -/- mice pair HF fed (2.2 g/day) for 6 weeks. Values are expressed as means ± SEM. *$p < 0.05$, comparing wildtype to CMKLR1-/- mice. N=3-4 mice/group.


Body Mass
Wildtype
CMKLR1 -/-
Weight (g)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Weeks on Diet

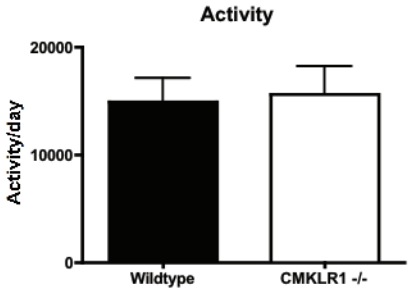
for all experimental groups (Figures 18, 19). However, two-way ANOVA analysis indicated that HF fed CMKLR1-/- mice had significantly lower oxygen consumption, carbon dioxide production, and energy expenditure as well as a significantly higher respiratory quotient compared to wildtype mice (Figure 19). However, post-hoc analysis did not indentify any individual time points as being significantly different. No significant effect of genotype was found in the LF fed groups using the same statistical analyses (Figure 18).

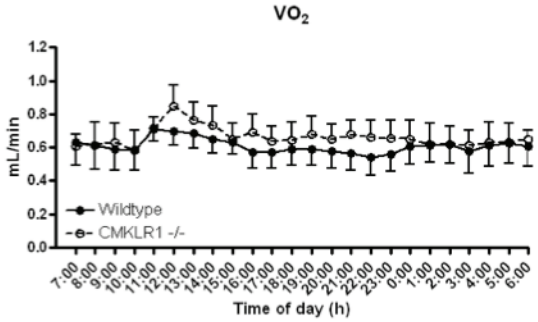
4.5.2 CMKLR1-/- Mice have Lower Blood Glucose and Serum Insulin, Leptin, and Adiponectin

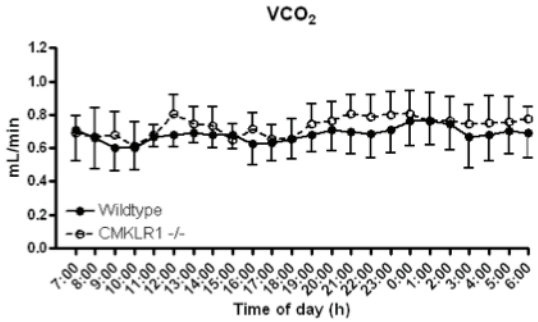
Fasting blood glucose levels were similar for CMKLR1-/- and wildtype mice when fed a LF diet for 6 or 24 weeks (Figure 20). However, when fed a HF diet for 24 weeks, fasting blood glucose levels were significantly reduced for CMKLR1-/- versus wildtype mice (Figure 21). Moreover, when fed a LF diet, wildtype mice at week 24 had significantly higher serum insulin levels than wildtype mice at week 6 (Figure 20). HF fed CMKLR1-/- mice at week 24 had significantly lower serum insulin levels than wildtype mice at week 24, and HF fed wildtype mice at week 24 had significantly higher serum insulin levels than wildtype mice at week 6 (Figure 21).

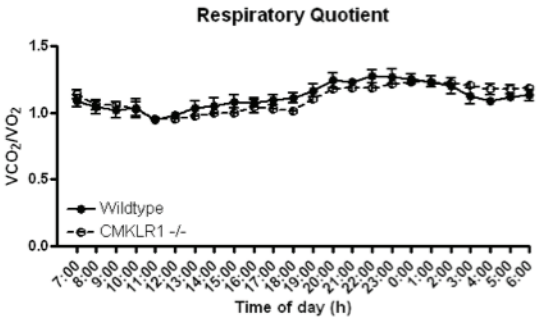
Serum levels of several adipokines including leptin and adiponectin correlate with adiposity and this is believed to underlie in part some of the undesirable metabolic changes that often coincide with obesity. Serum leptin levels were significantly lower in LF fed CMKLR1-/- mice from weeks 8 through 24, and HF fed CMKLR1-/- mice from weeks 4 through 12 (Figures 20, 21). Serum levels of the insulin-sensitizing adipokine adiponectin were significantly lower in CMKLR1-/- mice at week 0 (Figures 20, 21).

Figure 18. Low Fat Fed CMKLR1-/- have Similar Oxygen Consumption, Carbon Dioxide Production, Energy Expenditure, and Respiratory Quotient Compared to Wildtype Mice. Wildtype and CMKLR1-/- mice fed a LF diet for 6 weeks were individually housed in Panlab Physiocage 00 for 24 h with a fixed 12 h light (7:00 – 19:00):12 h dark (19:00 – 7:00) cycle to measure activity levels, oxygen consumption, carbon dioxide production , and respiratory quotient. The values are expressed as mean ± SEM. *$p < 0.05$, comparing wildtype to CMKLR1-/- mice. N=5-10 mice/group.


Activity
20000
10000
0
Activity/day
Wildtype
CMKLR1 -/-


VO2
1.2
1.0
0.8
0.6
0.4
0.2
0.0
mL/min
Wildtype
CMKLR1 -/-
7:00 8:00 9:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 21:00 22:00 23:00 0:00 1:00 2:00 3:00 4:00 5:00 6:00
Time of day (h)


VCO2
1.2
1.0
0.8
0.6
0.4
0.2
0.0
mL/min
Wildtype
CMKLR1 -/-
7:00 8:00 9:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 21:00 22:00 23:00 0:00 1:00 2:00 3:00 4:00 5:00 6:00
Time of day (h)


Respiratory Quotient
1.5
1.0
0.5
0.0
VCO2/VO2
Wildtype
CMKLR1 -/-
7:00 8:00 9:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 21:00 22:00 23:00 0:00 1:00 2:00 3:00 4:00 5:00 6:00
Time of day (h)

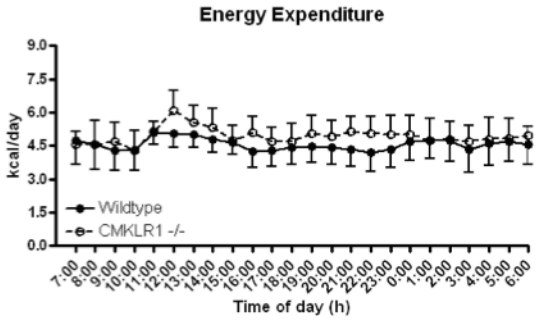
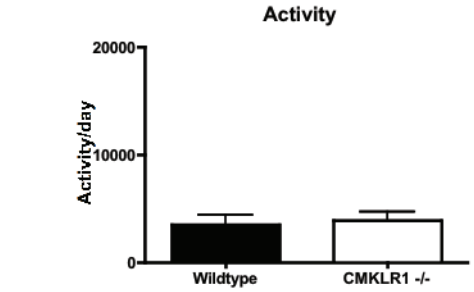
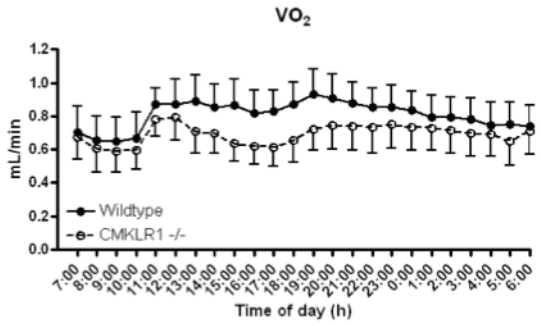
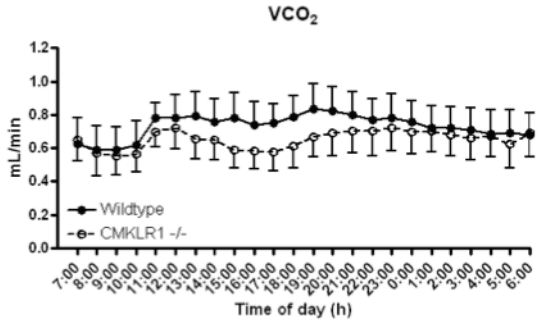
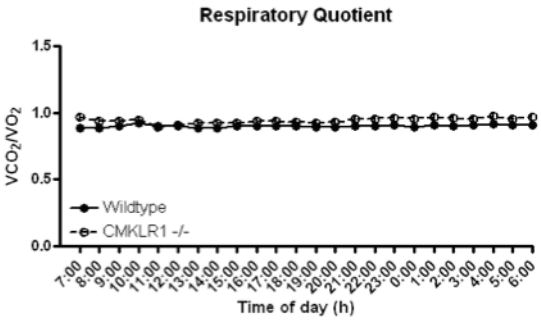

Energy Expenditure
9.0
7.5
6.0
4.5
3.0
1.5
0.0
kcal/day
Wildtype
CMKLR1 -/-
7:00 8:00 9:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 21:00 22:00 23:00 0:00 1:00 2:00 3:00 4:00 5:00 6:00
Time of day (h)

Figure 19. High Fat Fed CMKLR1-/- Mice have Lower Oxygen Consumption, Carbon Dioxide Production, and Energy Expenditure, and a Higher Respiratory Quotient Compared to Wildtype Mice. Wildtype and CMKLR1-/- mice fed a LF or HF diet for 6 weeks were individually housed in Panlab Physiocage 00 for 24 h with a fixed 12 h light (7:00 – 19:00):12 h dark (19:00 – 7:00) cycle to measure activity levels, oxygen consumption, carbon dioxide production, and respiratory quotient. The values are expressed as mean ± SEM. *$p < 0.05$, comparing wildtype to CMKLR1-/- mice. N=5-10 mice/group.


Activity
20000
10000
0
Activity/day
Wildtype
CMKLR1 -/-


VO2
mL/min
Wildtype
CMKLR1 -/-
Time of day (h)


VCO2
mL/min
Wildtype
CMKLR1 -/-
Time of day (h)


Respiratory Quotient
VCO2/VO2
Wildtype
CMKLR1 -/-
Time of day (h)

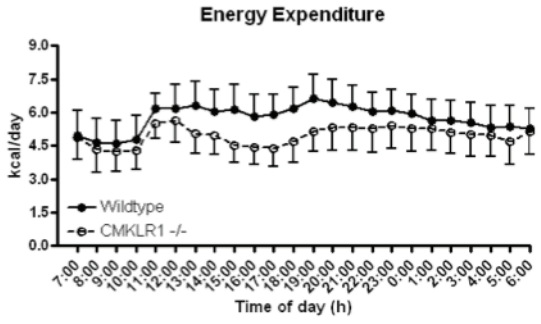
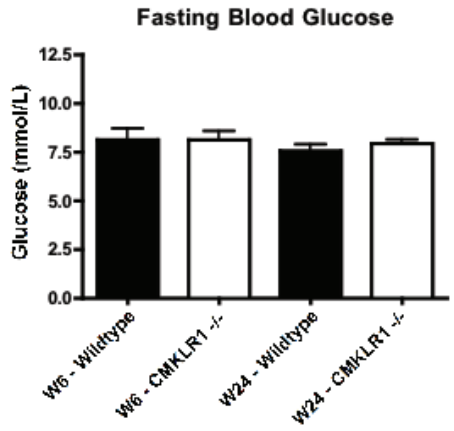
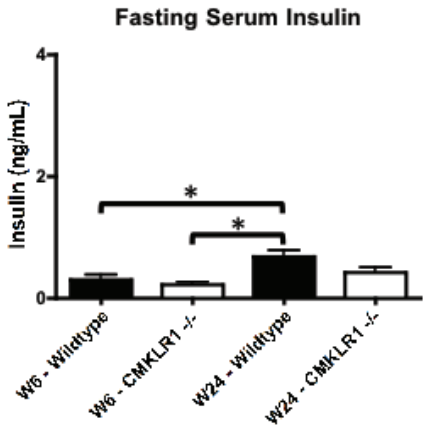
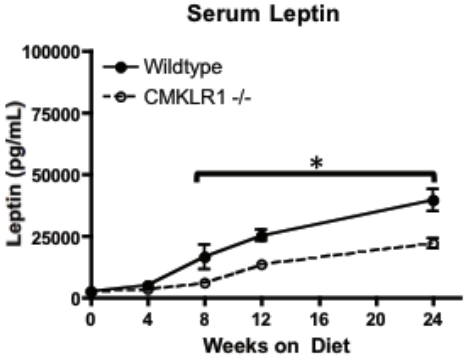
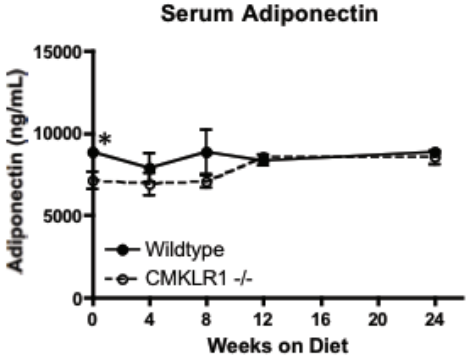

Energy Expenditure
kcal/day
Wildtype
CMKLR1 -/-
Time of day (h)

Figure 20. CMKLR1-Deficiency Reduces Serum Leptin and Adiponectin Levels in Low Fat Fed Mice. Fasting blood glucose and serum insulin levels were measured using a glucometer and ELISA respectively at week 6 and 24 in wildtype and CMKLR1-/- mice fed a LF diet. Serum leptin, adiponectin, and chemerin levels were quantified by ELISA at week 0, 4, 8, 12, and 24 in wildtype and CMKLR1-/- mice fed a LF diet.


Fasting Blood Glucose
Glucose (mmol/L)
12.5
10.0
7.5
5.0
2.5
0.0
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-


Fasting Serum Insulin
Insulin (ng/mL)
4
2
0
*
*
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-


Serum Leptin
Leptin (pg/mL)
100000
75000
50000
25000
0
Wildtype
CMKLR1 -/-
*
0
4
8
12
16
20
24
Weeks on Diet


Serum Adiponectin
Adiponectin (ng/mL)
15000
10000
5000
0
*
Wildtype
CMKLR1 -/-
0
4
8
12
16
20
24
Weeks on Diet

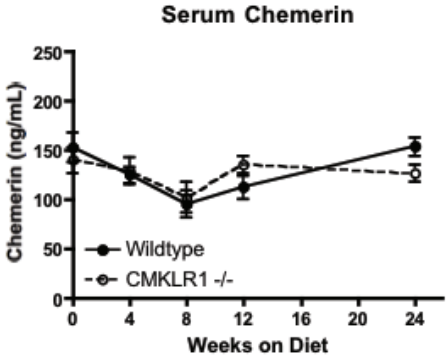
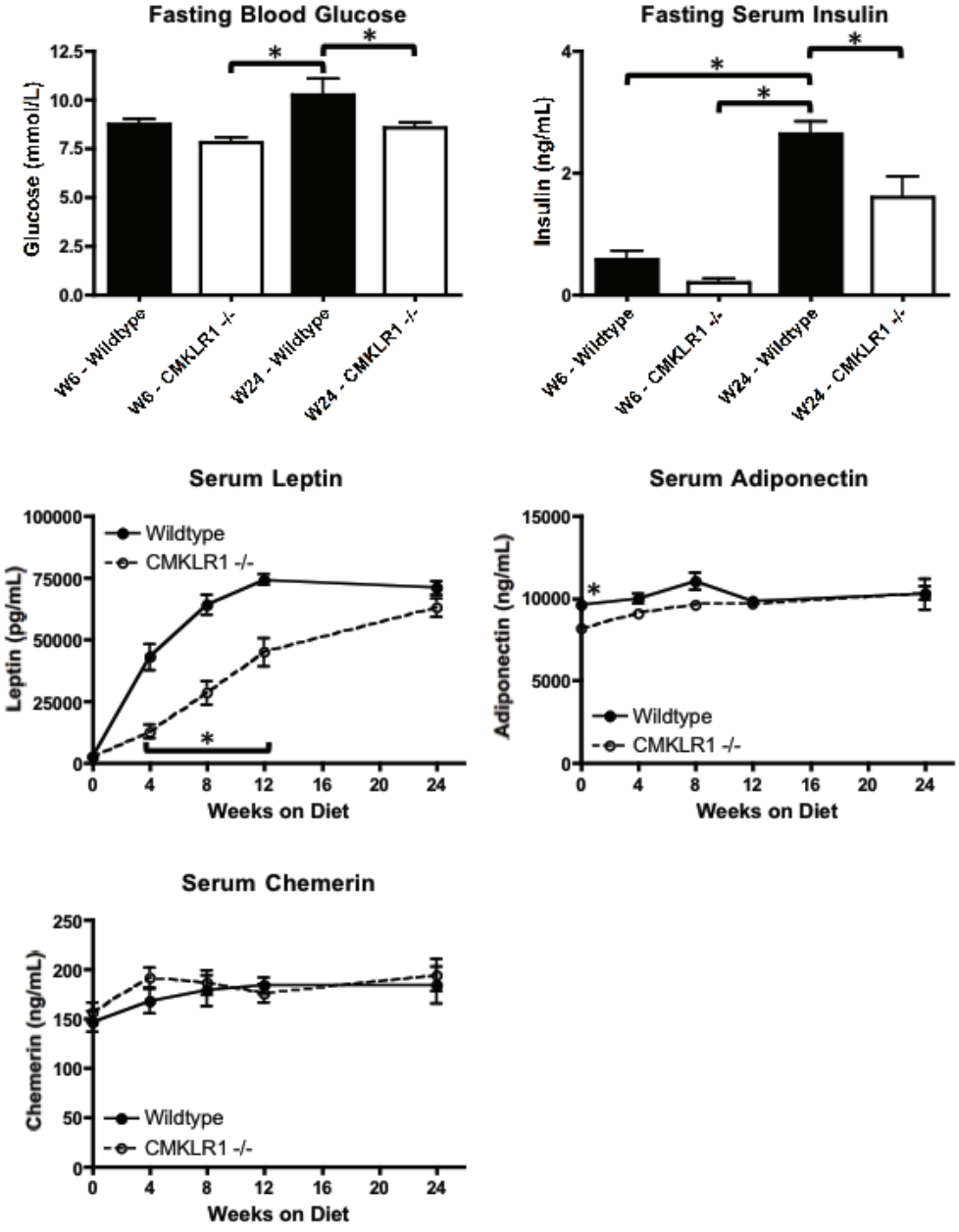

Serum Chemerin
Chemerin (ng/mL)
250
200
150
100
50
0
Wildtype
CMKLR1 -/-
0
4
8
12
16
20
24
Weeks on Diet

Figure 21. CMKLR1-Deficiency Reduces Blood Glucose and Serum Insulin, Leptin, and Adiponectin Levels in High Fat Fed Mice. Fasting blood glucose and serum insulin levels were measured using a glucometer and ELISA respectively at week 6 and 24 in wildtype and CMKLR1-/- mice fed a HF diet. Serum leptin, adiponectin, and chemerin levels were quantified by ELISA at week 0, 4, 8, 12, and 24 in wildtype and CMKLR1-/- mice fed a HF diet.


Fasting Blood Glucose
Glucose (mmol/L)
12.5
10.0
7.5
5.0
2.5
0.0
*
*
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-
Fasting Serum Insulin
Insulin (ng/mL)
4
2
0
*
*
*
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-
Serum Leptin
Leptin (pg/mL)
100000
75000
50000
25000
0
Wildtype
CMKLR1 -/-
*
0
4
8
12
16
20
24
Weeks on Diet
Serum Adiponectin
Adiponectin (ng/mL)
15000
10000
5000
0
*
Wildtype
CMKLR1 -/-
0
4
8
12
16
20
24
Weeks on Diet
Serum Chemerin
Chemerin (ng/mL)
250
200
150
100
50
0
Wildtype
CMKLR1 -/-
0
4
8
12
16
20
24
Weeks on Diet

Serum chemerin levels, while generally higher in HF fed mice, were similar between wildtype and CMKRL1 -/- mice, regardless of diet (Figures 20, 21).

4.5.3 CMKLR1-/- Mice Have Altered Pro-Inflammatory Cytokine Levels and Leukocyte Distributions in Liver And WAT

Obesity is commonly associated with hepatic steatosis due to impairment of lipid storage and metabolism in WAT. Wildtype and CMKLR1-/- mice fed a LF diet for 6 weeks displayed a similar liver histology, however at week 24, CMKLR1-/- mice had visibly less liver steatosis than wildtype mice (Figure 22). By comparison, no differences were observed between wildtype and CMKLR1-/- mice fed a HF diet for 6 or 24 weeks. Liver steatosis and obesity are commonly associated with inflammation. Thus, it was surprising that hepatic IL-6 mRNA levels were similar between CMKLR1-/- and wildtype mice regardless of age or diet (Figures 23, 24). However, within both the LF and HF fed groups, wildtype mice at week 24 had hepatic TNFα mRNA levels significantly greater than CMKLR1-/- mice at week 24 and wildtype mice at week 6 (Figures 23, 24). For WAT, no significant differences of IL-6 mRNA levels were observed within the LF fed groups (Figure 23). However, within the HF group, IL-6 mRNA levels were significantly higher for wildtype versus CMKLR1-/- mice at week 24 and wildtype mice at week 6 (Figure 24). Similar to liver tissue, within both the LF and HF groups, wildtype mice at week 24 had WAT TNFα mRNA levels that were significantly greater than CMKLR1-/- mice at week 24 and wildtype mice at week 6 (Figures 23, 24). Serum IL-6 levels were undetectable in LF fed mice at weeks 6 or 24 as well as HF fed mice at week 6, regardless of genotype (Figures 23, 24). After 24 weeks exposure to the HF diet,

Figure 22. CMKLR1-/- Mice have Less Hepatic Steatosis Than Wildtype Mice. Histological analysis of liver sections (H&E staining) from week 6 and 24 wildtype and CMKLR1-/- mice fed a LF or HF diet. Representative images are shown.

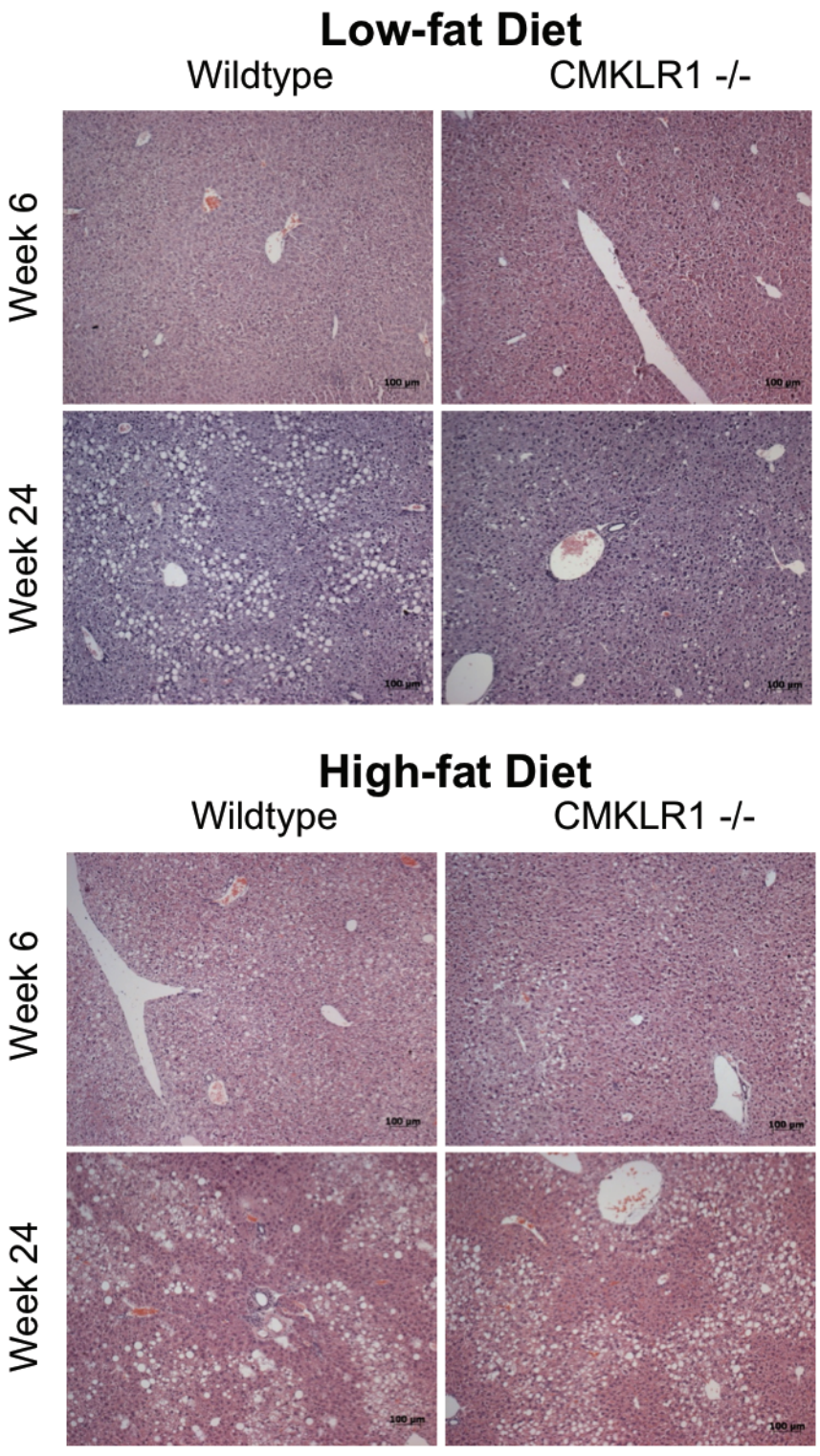

Low-fat Diet
Wildtype
CMKLR1 -/-
Week 6
Week 24
High-fat Diet
Wildtype
CMKLR1 -/-
Week 6
Week 24

Figure 23. IL-6 and TNFα Levels are Altered in Low Fat Fed CMKLR1-/- Mice. IL-6 and TNFα mRNA levels in liver and WAT as measured by quantitative real-time PCR, and serum IL-6 and TNFα levels quantified using ELISA at week 6 and 24 in wildtype and CMKLR1-/- mice fed a LF diet. Values are expressed as mean ± SEM. *$p < 0.05$, comparing wildtype to CMKLR1-/- mice. N=6-10 mice/group.

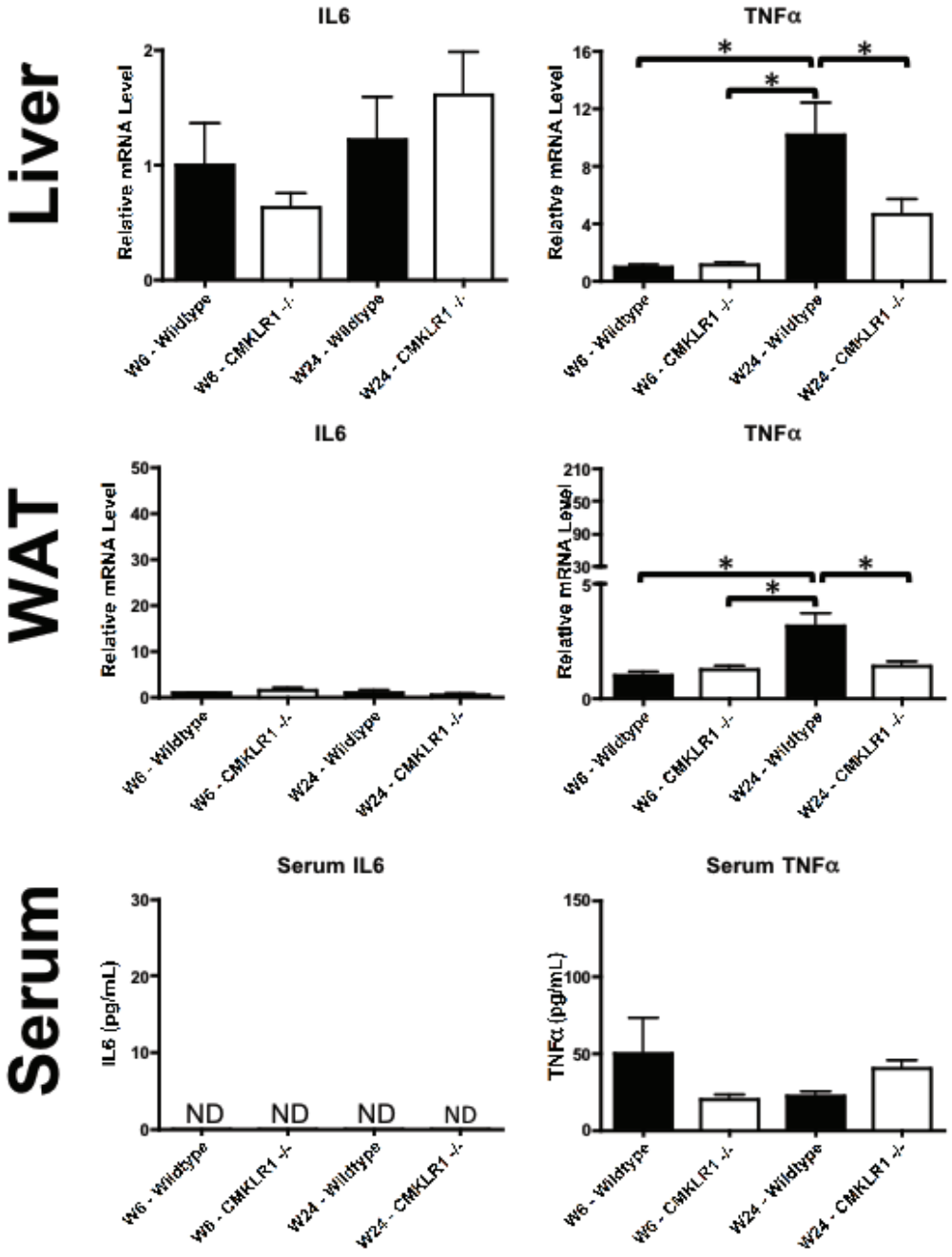
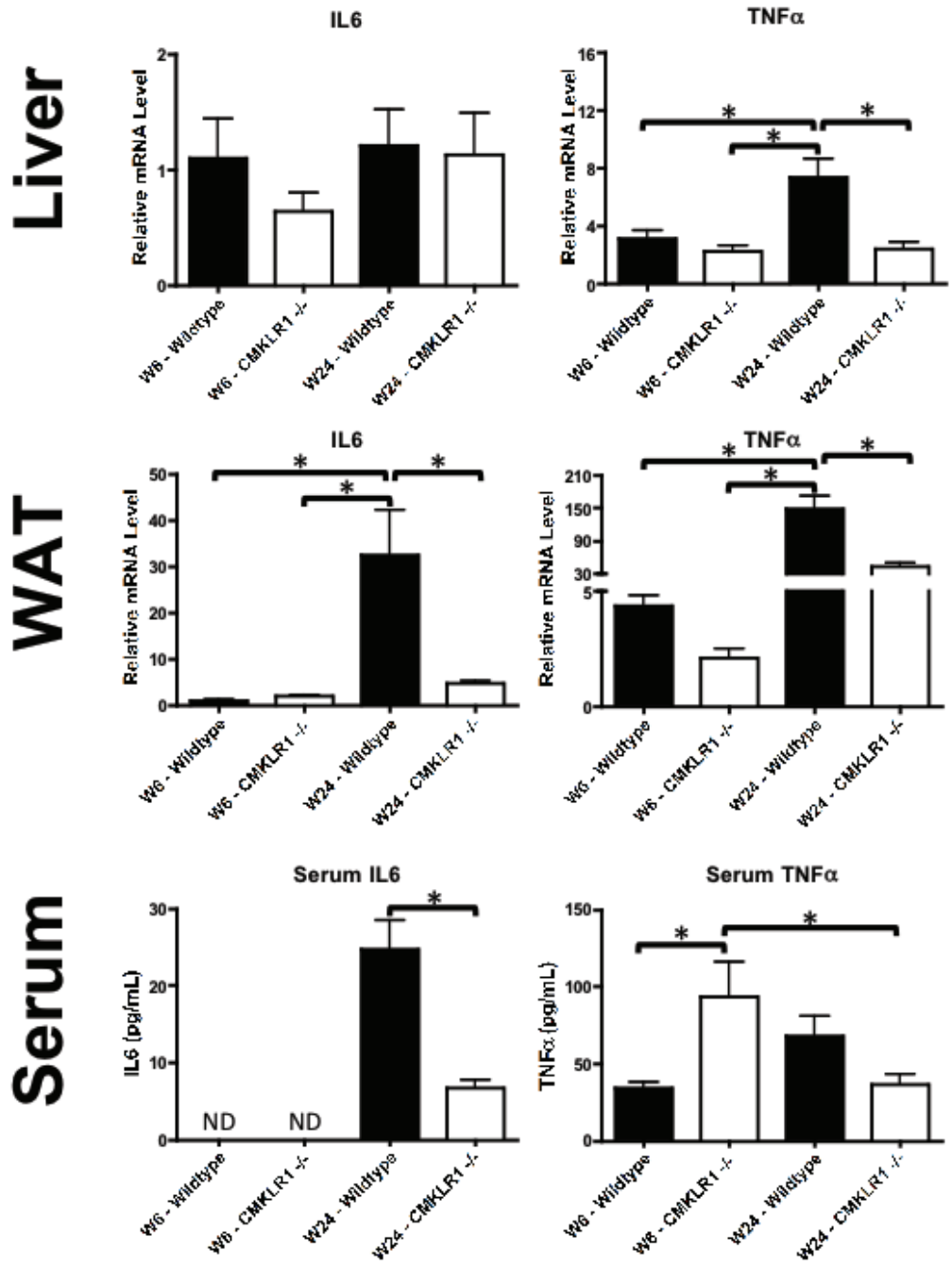

Liver
IL6
Relative mRNA Level
2
1
0
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-
TNFα
Relative mRNA Level
16
12
8
4
0
*
*
*
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-
WAT
IL6
Relative mRNA Level
50
40
30
20
10
0
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-
TNFα
Relative mRNA Level
210
150
90
30
5
0
*
*
*
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-
Serum
Serum IL6
IL6 (pg/mL)
30
20
10
0
ND
ND
ND
ND
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-
Serum TNFα
TNFα (pg/mL)
150
100
50
0
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-

Figure 24. IL-6 and TNFα Levels are Altered in High Fat Fed CMKLR1-/- Mice. IL-6 and TNFα mRNA levels in liver and WAT as measured by quantitative real-time PCR, and serum IL-6 and TNFα levels quantified using ELISA at week 6 and 24 in wildtype and CMKLR1-/- mice fed a HF diet. Values are expressed as mean ± SEM. *$p < 0.05$, comparing wildtype to CMKLR1-/- mice. N=6-10 mice/group.


Liver
WAT
Serum
IL6
TNFα
Relative mRNA Level
Serum IL6
Serum TNFα
IL6 (pg/mL)
TNFα (pg/mL)
ND
ND
W6 - Wildtype
W6 - CMKLR1 -/-
W24 - Wildtype
W24 - CMKLR1 -/-

CMKLR1-/- mice had significantly lower serum IL-6 levels than wildtype mice (Figure 24). No significant differences in serum TNFα levels were found within the LF fed group (Figure 23). However, within the HF fed group, CMKLR1-/- mice at week 6 hadsignificantly higher serum TNFα levels compared to wildtype mice at week 6, and CMKLR1-/- mice at week 24 (Figure 24).

Given that chemerin is a chemoattractant for subsets of dendritic cells, macrophages, and NK cells, immune cell infiltration in the liver and WAT was assessed. In the liver, the percentage of dendritic cells was significantly lower in CMKLR1-/- mice compared to wildtype mice within the LF fed group (24 weeks; Table 3). In contrast, no significant differences were observed for the hepatic immune cell population of HF fed mice (Table 4). In WAT, both LF and HF fed CMKLR1-/- mice had a significantly lower percentage of CD3+ T cells, and a significantly greater percentage of natural killer cells compared to wildtype mice (Tables 3, 4).

4.5.4 Reduced Glucose Tolerance and Insulin Secretion in CMKLR1-/- Mice

To investigate the impact of CMKLR1 gene deletion on glucose homeostasis, insulin sensitivity and glucose tolerance tests (GTT) were performed with wildtype and CMKLR1-/- mice fed a LF or HF diet. A single bolus of insulin produced a similar decline in blood glucose levels for both wildtype and CMKLR1-/- mice at 6 and 24 week time points regardless of diet, indicating that CMKLR1 deletion did not impact insulin sensitivity (Figures 25, 26). However, CMKLR1-/- mice were significantly more glucose intolerant than wildtype mice at week 6 when fed a LF diet (Figure 25) and at week 24 when fed a HF diet (Figure 26). In LF fed mice, a significant difference in serum insulin

Table 3. CMKLR1 Loss Alters Immune Cell Infiltration in Liver and White Adipose Tissue in Low Fat Fed Mice. Flow cytometry analysis of liver and WAT from wildtype and CMKLR1-/- mice fed a LF diet for 24 weeks. Values are expressed as mean ± SEM. ***p < 0.05**, comparing wildtype to CMKLR1-/- mice. N=3-5.

Liver								
Cell Type	Wildtype (%)			CMKLR1 -/- (%)			P
Neutrophils	0.34	±	0.09	0.14	–	0.03	0.14
Macrophages	1.76	±	0.91	3.84	±	1.04	0.20
Dendritic Cells	3.13	±	0.19	1.70	±	0.41	**0.03**
B cells	15.95	±	7.70	37.44	±	10.60	0.19
CD3+ T cells	50.52	±	8.59	29.55	±	6.03	0.09
Natural Killer T cells	1.75	±	0.33	1.81	±	0.30	0.89
Natural Killer cells	7.46	±	0.49	8.12	±	1.39	0.67
White Adipose Tissue							
Cell Type	Wildtype (%)			CMKLR1 -/- (%)			P
Neutrophils		–			–		–
Macrophages	8.21	±	1.61	7.34	±	0.99	0.65
Dendritic Cells	1.58	±	0.50	2.33	±	0.97	0.52
B cells	2.71	±	1.24	6.61	±	3.73	0.36
CD3+ T cells	54.62	±	6.87	30.98	±	6.59	**0.05**
Natural Killer T cells	1.32	±	0.55	1.80	±	0.64	0.59
Natural Killer cells	6.61	±	1.73	15.08	±	1.37	**0.01**

Table 4. CMKLR1 Loss Alters Immune Cell Infiltration in Liver and White Adipose Tissue in High Fat Fed Mice. Flow cytometry analysis of liver and WAT from wildtype and CMKLR1-/- mice fed a HF diet for 24 weeks. Values are expressed as mean ± SEM. ***p < 0.05**, comparing wildtype to CMKLR1-/- mice. N=3-5.

Liver								
Cell Type	Wildtype (%)			CMKLR1 -/- (%)			P
Neutrophils	1.74	±	0.90	0.06	±	0.02	0.13
Macrophages	6.42	±	2.83	5.59	±	0.90	0.77
Dendritic Cells	2.99	±	0.62	2.43	±	0.45	0.48
B cells	14.25	±	7.37	32.69	±	4.92	0.07
CD3+ T cells	34.19	±	7.94	32.90	±	4.18	0.88
Natural Killer T cells	2.69	±	0.73	2.43	±	0.37	0.75
Natural Killer cells	7.18	±	1.13	7.17	±	0.67	1.00
White Adipose Tissue							
Cell Type	Wildtype (%)			CMKLR1 -/- (%)			P
Neutrophils		–			–		–
Macrophages	10.23	±	4.99	13.15	±	3.43	0.63
Dendritic Cells	1.91	±	0.29	2.98	±	1.59	0.58
B cells	12.05	±	1.54	7.79	±	1.47	0.12
CD3+ T cells	59.38	±	6.14	40.68	±	3.51	**0.04**
Natural Killer T cells	2.03	±	0.17	4.33	±	0.79	0.07
Natural Killer cells	5.33	±	0.50	14.52	±	2.04	**0.02**

Figure 25. CMKLR1 Loss is Associated with Glucose Intolerance and Decreased Glucose-Stimulated Insulin Secretion in Low Fat Fed Mice. Insulin sensitivity tests, glucose tolerance tests, serum insulin levels during glucose tolerance tests, and area under the curve analysis of serum insulin levels from LF fed wildtype and CMKLR1-/- mice at week 6 and 24. Values are expressed as mean ± SEM. *$p < 0.05$, comparing wildtype to CMKLR1-/- mice. N=5-10 mice/group.

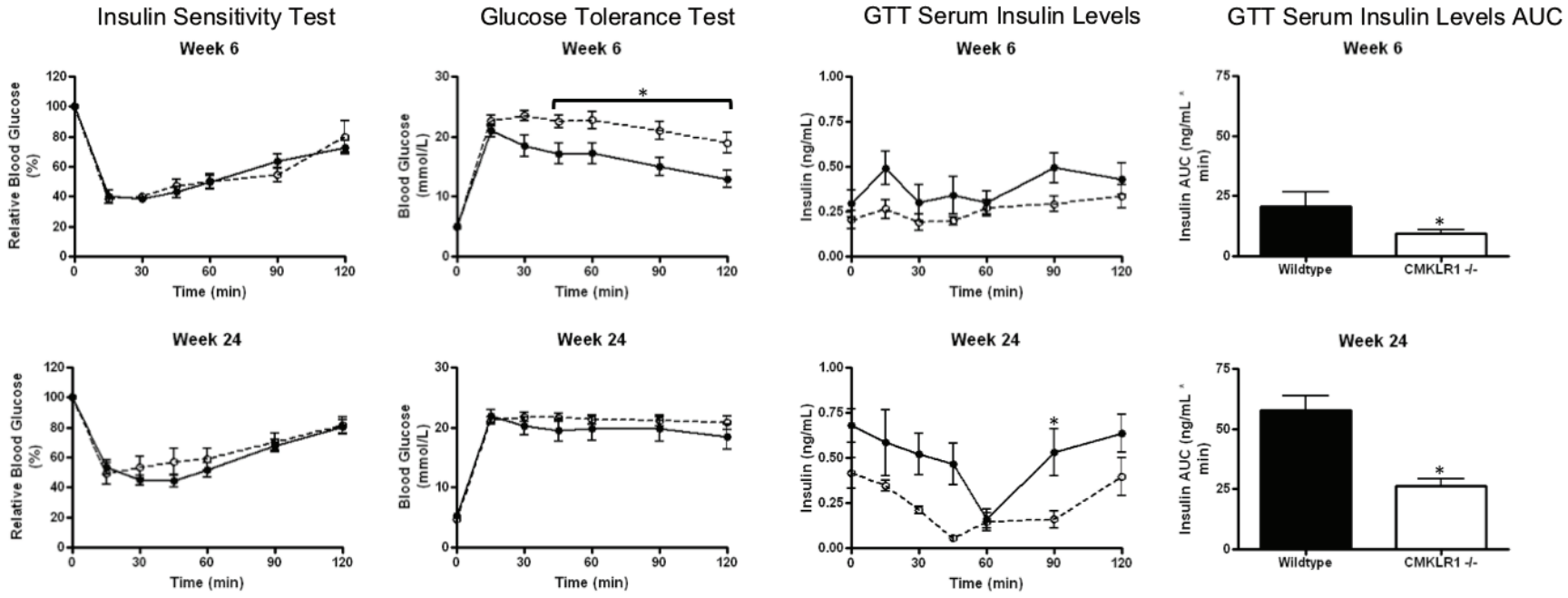
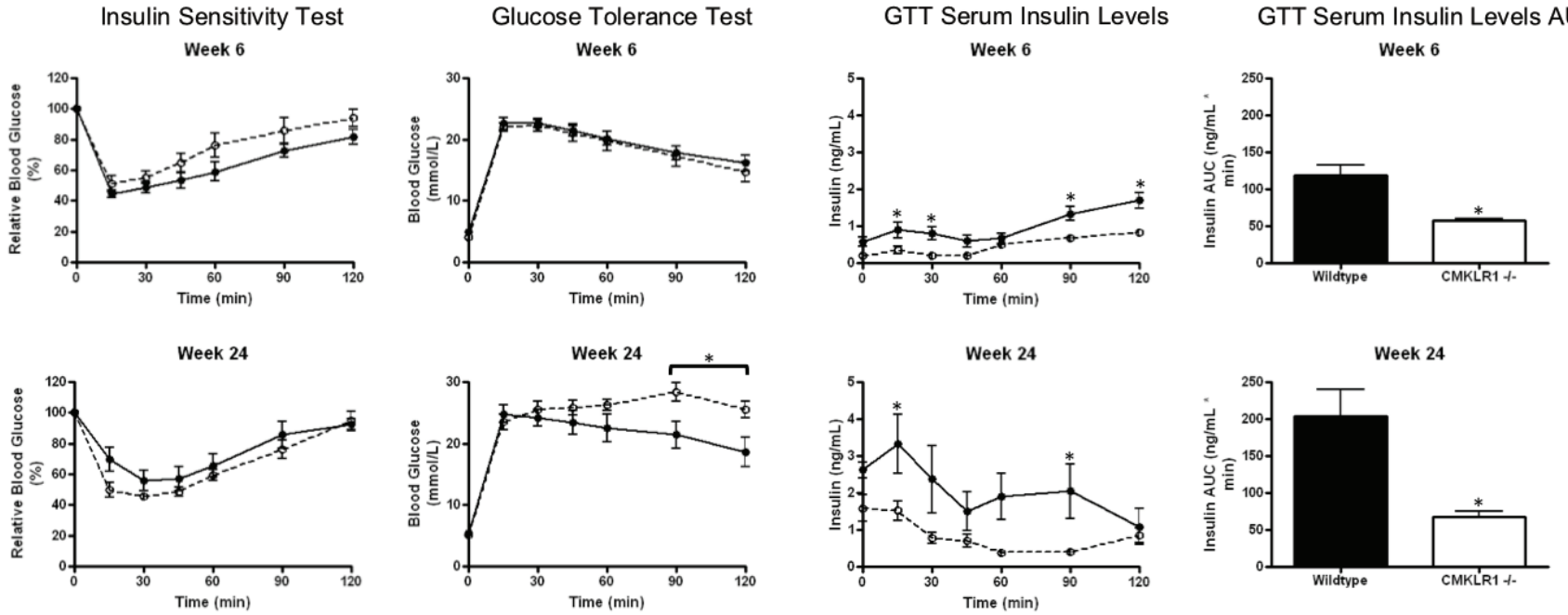

Insulin Sensitivity Test
Week 6
Relative Blood Glucose (%)
Time (min)
Week 24
Relative Blood Glucose (%)
Time (min)
Glucose Tolerance Test
Week 6
Blood Glucose (mmol/L)
Time (min)
Week 24
Blood Glucose (mmol/L)
Time (min)
GTT Serum Insulin Levels
Week 6
Insulin (ng/mL)
Time (min)
Week 24
Insulin (ng/mL)
Time (min)
GTT Serum Insulin Levels AUC
Week 6
Insulin AUC (ng/mL * min)
Wildtype
CMKLR1 -/-
Week 24
Insulin AUC (ng/mL * min)
Wildtype
CMKLR1 -/-

Figure 26. CMKLR1 Loss is Associated with Glucose Intolerance and Decreased Glucose-Stimulated Insulin Secretion in High Fat Fed Mice. Insulin sensitivity tests, glucose tolerance tests, serum insulin levels during glucose tolerance tests, and area under the curve analysis of serum insulin levels from HF fed wildtype and CMKLR1-/- mice at week 6 and 24. Values are expressed as mean ± SEM. *$p < 0.05$, comparing wildtype to CMKLR1-/- mice. N=5-10 mice/group.


Insulin Sensitivity Test
Week 6
Relative Blood Glucose (%)
Time (min)
Week 24
Relative Blood Glucose (%)
Time (min)
Glucose Tolerance Test
Week 6
Blood Glucose (mmol/L)
Time (min)
Week 24
Blood Glucose (mmol/L)
Time (min)
GTT Serum Insulin Levels
Week 6
Insulin (ng/mL)
Time (min)
Week 24
Insulin (ng/mL)
Time (min)
GTT Serum Insulin Levels AUC
Week 6
Insulin AUC (ng/mL * min)
Wildtype
CMKLR1 -/-
Week 24
Insulin AUC (ng/mL * min)
Wildtype
CMKLR1 -/-

levels during the GTT was found between wildtype and CMKLR1-/- mice only at the 90 min sample point for animals maintained on the diet for 24 weeks (Figure 25). However, area under the curve (AUC) analysis of these data indicated that the total exposure to insulin was significantly lower in CMKLR1-/- mice compared to wildtype mice after 6 or 24 weeks on the LF diet. In HF fed mice, week 6 and week 24 GTT serum insulin levels were significantly lower in CMKLR1-/- mice at the 15, 30, 90, and 120 min, and the 15 and 90 min sample points, respectively (Figure 26). Consistent with the LF group, the insulin AUC was significantly lower in HF fed CMKLR1-/- mice compared to wildtype mice after 6 and 24 weeks on the HF diet. To determine if the reduced glucose tolerance in CMKLR1-/- mice reflected a decrease in tissue glucose uptake, we performed *in vivo* tissue glucose uptake experiments. Consistent with GTT results, CMKLR1 null mice fed the LF diet for 6 weeks had significantly lower total tissue, skeletal muscle and WAT glucose uptake compared to wildtype controls (Figure 27). Also, consistent with the GTT results, while there was a general trend towards reduced tissue glucose in HF versus LF mice overall, there was no significant difference between the genotypes after 6 weeks on the HF diet.

4.6 Discussion

Herein, we report for the first time that loss of CMKLR1, a receptor for the adipokine chemerin, decreases food consumption, weight gain, and adiposity in mice. Consistent with a leaner phenotype, CMKLR1-/- mice have lower fasting blood glucose and serum insulin levels. CMKLR1 loss also protected against hepatic steatosis, and resulted in a decrease in the mRNA levels of the pro-inflammatory cytokines TNFα and IL-6 in WAT. However, our novel findings have also revealed a complex phenotype

Figure 27. CMKLR1 Loss Decreases *In Vivo* Tissue Glucose Uptake. Total, liver, skeletal muscle (SKM) and white adipose tissue (WAT) glucose uptake in wildtype and CMKLR1-/- mice. Mice were injected IP with glucose and 2-[1,2-^{3}H(N)]deoxy-D-glucose, and blood samples were collected over a 1 hour period. Values are expressed as mean ± SEM. *$p < 0.05$, comparing wildtype to CMKLR1-/- mice within the diet. N=5-10 mice/group.


Low-fat Diet
Glucose Uptake
DPM/mg/GSA
0.10
0.08
0.06
0.04
0.02
0.00
*
*
*
Total
Liver
SKM
WAT
Wildtype
CMKLR1 -/-


High-fat Diet
Glucose Uptake
DPM/mg/GSA
0.10
0.08
0.06
0.04
0.02
0.00
Total
Liver
SKM
WAT
Wildtype
CMKLR1 -/-

whereby the apparently beneficial effects of CMKLR1-deficiency *in vivo* are balanced by potentially deleterious changes in glucose tolerance associated with decreased glucose-stimulated insulin secretion and tissue glucose uptake.

Previous studies have shown that loss of CMKLR1 expression/function impairs adipogenesis in cell culture models [228, 265]. Consistent with these findings, CMKLR1 loss *in vivo* resulted in a lower total body mass, primarily due to significantly lower fat mass. CMKLR1-/- mice had markedly lower food consumption compared to wildtype controls. This unanticipated finding suggests that the loss of CMKLR1 affects other regulators of appetite, or that chemerin is an orexigenic adipokine acting through CMKLR1. Activity levels were similar in wildtype and CMKLR1-/- mice, indicating that energy expenditure through locomotor activity was not a factor contributing to the decreased body mass. While the indirect calorimetry indicated a subtle alteration of metabolic phenotype for HF fed CMKLR1-/- mice, interpretation of these data is confounded by the differences in food consumption as the reduced energy expenditure could simply reflect a reduction in energy intake. However, paired feeding showed that limiting wildtype daily food consumption to CMKLR1 -/- levels eliminated the previously observed difference in weight gain, and resulted in CMKLR1 -/- mice having a greater body mass than wildtype mice, again indicating a subtle alteration of metabolic phenotype in CMKLR1 -/- mice. Thus, at present we can only conclude that the reduced body weight and adiposity associated with CMKLR1 loss primarily derives from differences in feeding, and possibly to some extent from altered metabolic phenotype and reduced adipogenesis. Further studies are clearly required to elucidate the specific role of chemerin/CMKLR1 signaling in appetite regulation.

Obesity-associated imbalances in adipokine secretion are believed to contribute to the risk for development of various deleterious metabolic changes characteristic of metabolic syndrome [17, 18]. We found significantly lower serum leptin levels in both LF and HF fed CMKLR1-/- mice, a finding consistent with the well established positive correlation of leptin levels with fat mass [167]. However, leptin is also an adipocyte-derived anorexigenic factor [305] and despite having lower serum leptin levels throughout the study, food consumption was consistently lower in CMKLR1-/- mice. This reiterates the need for further studies to examine the role of CMKLR1 in appetite regulation and any mechanistic interactions between chemerin and leptin signaling. While the serum levels of adiponectin were significantly lower in CMKLR1-/- mice at baseline, the lack of persistent difference throughout the remainder of the study indicates that alterations in the secretion of this insulin-sensitizing adipokine did not contribute substantially to the metabolic phenotype of the knockout mice.

In addition to the growing body of evidence linking chemerin/CMKLR1 signaling to adiposity and energy metabolism, this signaling pathway also has physiological relevance to immune function. CMKLR1 is expressed by a number of immune cells, including plasmacytoid DCs, myeloid DCs, macrophages and natural killer (NK) cells [220, 226]. Following activation, these cells synthesize and secrete a number of pro-inflammatory cytokines, including IL-6 and TNFα. Interestingly, hepatic mRNA levels for the pro-inflammatory cytokine TNFa were reduced in the livers of CMKLR1-/- versus wildtype mice fed the LF diet for 24 weeks, a finding consistent with the reduced hepatic steatosis in these mice. It is well established that obesity is associated with chronic low-grade inflammation [194]. Interestingly, WAT IL-6 mRNA levels were significantly

lower in week 24 HF fed CMKLR1-/- mice, and similar to the liver, WAT TNFα mRNA levels were significantly lower in week 24 LF and HF fed CMKLR1-/- mice. Consistent with WAT mRNA, serum IL-6 levels were significantly lower in week 24 HF fed CMKLR1-/- mice while TNFα levels were not reflective of the mRNA levels of liver or WAT. Serum TNFα concentrations were similar in LF fed mice, but were significantly higher in week 6 HF fed CMKLR1-/- mice. This suggests that tissues other than liver and WAT are responsible for the observed increase in TNFα levels. Flow cytometry of liver leukocytes from CMKLR1-/- mice fed a LF diet for 24 weeks showed a decrease in dendritic cell infiltration compared to wildtype mice, an effect not observed in HF fed counterparts. Liver dendritic cells can contribute to hepatic inflammation by activating NK cells and T cells [306]. Further analysis of WAT from LF and HF fed mice showed significantly less CD3+ T cells in CMKLR1-/- compared to wildtype and a significant increase in NK cells in CMKLR1-/- mice. WAT infiltration by natural killer cells is elevated in mice lacking mature T or B cells when fed a HF diet [307]. Given the reduction in WAT CD3+ T cells in CMKLR1 KO mice, the increase in natural killer cells may reflect a similar finding. Taken together, our data provide novel evidence implicating CMKLR1 in the recruitment of various immune cells to liver and WAT.

A growing body of human clinical data indicates that serum chemerin levels are elevated with obesity and correlate with many facets of metabolic syndrome, including insulin resistance and type II diabetes [229, 241, 242]. Consistent with this, recombinant chemerin administration exacerbates glucose intolerance in obese/diabetic mice [279] and chronic overexpression of chemerin causes glucose intolerance by inducing skeletal muscle insulin resistance in mice [292]. Our data are also consistent with a role for

chemerin/CMKLR1 in energy homeostasis and indicate an influence glucose-stimulated insulin secretion and insulin-stimulated glucose uptake in skeletal muscle and WAT. ERK activation has been reported to regulate ATP-sensitive potassium channels that play an important role in controlling pancreatic insulin secretion [308] and it is well established that Ca^{+2} influx activates protein motors and kinases that mediate exocytosis of insulin-containing vesicles [47, 49]. CMKLR1 activation has been reported to induce intracellular calcium release and phosphorylation of ERK kinases [219, 228] and thus may impact insulin secereton through one or both of these intracellular signaling events. Interestingly, LF and HF fed mice age, CMKLR1-/- mice remain lean compared to wildtype mice and exhibit lower fasting blood glucose and serum insulin levels. The CMKLR1-/- mice also exhibit generally lower circulating concentrations and tissue expression levels of pro-inflammatory cytokines. Yet despite this overtly "healthier" phenotype, HF fed CMKLR1-/- mice were more glucose intolerant than wildtype mice after 12 and 24 weeks on the diet. Similarly, individuals exist who are not obese on the basis of height and weight, but are predisposed to glucose intolerance and type II diabetes. These individuals are classified as metabolically obese normal weight [309]. Therefore, the lean phenotype in conjunction with glucose intolerance suggests that CMKLR1-/- mice may display a similar metabolically obese normal weight phenotype.

In summary, we provide evidence for a complex phenotype associated with CMKLR1 loss that is characterized by lower food consumption and reduced body mass and adiposity, coincident with worsened glucose tolerance as well as decreased glucose stimulated insulin secretion and tissue glucose uptake. Moreover, our data also demonstrate that CMKLR1 influences the expression of pro-inflammatory cytokines and composition of leukocytes present in the liver and WAT. Thus, characterization of the

function of CMKLR1 in satiety, weight gain, and glucose tolerance has the potential to lead to novel therapeutic approaches for the treatment of obesity and type II diabetes.

Chapter V: General Discussion and Conclusions

The increasing prevalence of obesity is a major public health concern and a substantial burden on the health care resources of Canada and other highly industrialized societies of North America and Europe. In addition to the direct health impact of obesity, this condition is associated with a variety of metabolic derangements that increase the risk for developing a number of diseases including hypertension, cardiovascular disease, and type II diabetes [11, 12]. Despite the clear link between obesity and the development of these comorbidities, the mechanisms responsible for this relationship are not fully understood. Historically, adipose tissue has been regarded primarily as an energy storage organ. However, the discovery of leptin in the 1990's as an adipose tissue derived signaling molecule, or an adipokine, revolutionized our understanding of adipose tissue function. Since this discovery, the number of known adipokines has expanded rapidly, and now includes pro-inflammatory cytokines and cytokine-related proteins, complement and complement-related proteins, fibrinolytic proteins, and proteins of the renin–angiotensin system, as well as a variety of other biologically active proteins with hormone-like actions such as leptin and adiponectin. Adipokines have been shown to regulate a number of biological functions, including adipocyte differentiation and function, appetite and energy balance, glucose tolerance and insulin sensitivity, and inflammation. The synthesis and secretion of many adipokines is dynamic and modifiable, with levels of pro-inflammatory/pro-diabetic adipokines commonly increasing with obesity, and levels of anti-inflammatory/anti-diabetic adipokines decreasing with obesity [16]. This change in the synthesis and secretion of adipokines with obesity contributes to the development of obesity and obesity-related diseases [17-19].

In 2007, our laboratory identified a novel adipokine known as chemerin. Experimental evidence supports a role for chemerin in various aspects of human physiology and pathophysiology, including obesity, inflammation, and glucose homeostasis. Studies have shown that serum chemerin levels are elevated in obesity/diabetes, however, the precise role of chemerin in these disorders remains unclear. Since the initial identification of chemerin as ligand for CMKLR1 [219], a tremendous amount of information has emerged regarding this protein. While much remains to be learned regarding the physiologic/pathophysiologic relevance of chemerin, two parallel themes have clearly emerged – inflammation and metabolism. Therefore, we have proposed that chemerin contributes to the pathology of insulin resistance through the regulation and modulation of glucose homeostasis in white adipose, skeletal muscle, and liver tissue. Furthermore, we hypothesize that the loss of the chemerin receptor CMKLR1 *in vivo* would reduce adiposity and impact glucose homeostasis. Herein, we have shown that chemerin, similar to other adipokines, regulates a number of biological functions, including appetite and energy balance, adiposity, glucose homeostasis, and inflammation.

Similar to other pro-inflammatory adipokines, including TNFα and IL-6, the majority of human data indicates that serum chemerin levels are elevated in obesity/diabetes [229, 242, 271, 272, 276, 302-304]. Consistent with these studies, we found that total serum chemerin levels were elevated in mouse models of obesity and diabetes. Unlike other adipokines such as adiponectin and leptin, which are almost exclusively expressed in adipose tissue, chemerin is also highly expressed in liver and has low levels of expression in other tissues [228]. However, further mRNA and protein analysis of obese tissues suggested that elevated serum chemerin levels were the result of an increase in chemerin secretion from WAT, not liver, and were caused by changes in

post-translational processes (i.e. proteolytic processing, secretion). In support of this, the pro-inflammatory/pro-diabetic adipokine TNFα, which has been shown to be elevated in obesity, increases the synthesis and secretion of chemerin from adipocytes but not hepatocytes [280].

Several adipokines have been shown to be secreted as trimers or oligomers, or undergo proteolytic processing to produce an active peptide. Adiponectin circulates as several multimeric complexes, including homodimers, hexamers, and high molecular weight complexes, which have different affinities for the adiponectin receptors AdipoR1 and AdipoR2, and thus elicit different effects [173, 174, 294]. In addition, acylation-stimulating protein (ASP), an adipokine with a major role in the stimulation of triacylglyceride metabolism and glucose uptake [310-312], is a cleavage product of the complement C3 protein. Cleavage requires interaction between the alternative pathway proteins C3 and factor B, and the serine protease adipsin [313]. Similarly, the inactive prochemerin protein has low biological activity and requires further extracellular C-terminal processing by plasmin, carboxypeptidases, or serine proteases of the coagulation, fibrinolytic, and inflammatory cascades to produce several C-terminal derivatives with varying levels of biological activity [219-221, 226, 230]. We determined that in addition to elevated serum total chemerin protein levels, bioactive chemerin levels were also elevated in mouse models of obesity. Furthermore, the concentration of bioactive chemerin levels was significantly higher than serum levels of total chemerin protein. Since studies have shown that several chemerin derivatives have different levels of activity, serum levels of bioactive chemerin are likely higher than the concentration of total chemerin protein because there are chemerin derivatives in the serum with activities similar to or greater than recombinant mouse chemerin used to generate the standard

curve for the assay used to measure bioactive chemerin levels. This finding not only stresses the importance of measuring both bioactive and total chemerin levels, but also shows that future studies are required to determine which forms of chemerin are found in tissues of interest, which enzymes are present that are capable of proteolytically cleaving chemerin, and which forms are responsible for observed differences in adipogenesis, glucose uptake, and other cellular processes. Therefore, we have shown that chemerin secretion is dynamic and modifiable, with serum levels increasing with obesity, suggesting that this change in secretion may contribute to obesity and the development of obesity-associated diseases.

Many adipokines have multiple receptors with different expression profiles and ligand affinity. For example, adiponectin binds to AdipoR1 and AdipoR2. AdipoR1 is expressed primarily in brain, heart, kidney, liver, lung, spleen and testes, and has a high affinity for globular adiponectin, and a low affinity for full-length adiponectin [174, 294]. AdipoR2 is mostly highly expressed in liver, and has intermediate affinity for globular and full length adiponectin [174, 294]. The numerous tissues that express adiponectin receptors, combined with varying receptor affinity for different forms of adiponectin, results in a wide spectrum of effects for this adipokine. Similarly, chemerin binds to three known receptors: CMKLR1, CCRL2, and GPR1. The majority of studies to date have described the physiological functions of chemerin elicited through activation of CMKLR1. CCRL2 appears to be a non-signaling receptor that binds chemerin and increases the local concentration of the peptide [221, 222], and studies examining the role of GPR1 are currently lacking [223, 224]. Herein, we have shown that these three receptors have different expression profiles in WAT, SKM, and liver. CMKLR1 is most highly expressed in WAT, CCRL2 expression is highest in SKM with moderate levels of

expression in WAT and liver, and GPR1 expression is highest in SKM, with moderate expression in WAT. In addition, we have consistently shown that CMKLR1 mRNA levels increase WAT, and decrease in SKM of obese mice. The numerous tissues that express chemerin receptors suggest that chemerin has a wide spectrum of effects, and a change in receptor expression with obesity indicates that these effects change with disease state. Unfortunately, while one study suggests that chemerin has a higher affinity for GPR1 than CMKLR1, studies examining the affinity of different chemerin derivatives with the three known chemerin receptors are lacking [222]. Future studies examining the expression profile of these receptors in additional tissues of both lean and obese mice, as well as ligand affinity for the receptors will be essential in evaluating the widespread effects of chemerin.

Adipokines affect many physiological processes, including appetite and energy balance [14, 15]. For example, leptin crosses the blood-brain barrier and serves as an efferent signal to the central nervous system from adipose tissue capable of altering food intake, body weight, energy expenditure, and neuroendocrine function [160]. By acting on specialized hypothalamic pathways, leptin decreases the activity of neurons that stimulate appetite and inhibit energy expenditure, and increases the activity of neurons that inhibit appetite and increase energy expenditure. Leptin also increases the expression of the anorexigenic factors and decreases the expression of the orexigenic factors [161]. Mutation of the leptin receptor ObRb results in massive obesity, insulin resistance, and diabetes. Herein, we report for the first time a potential role for chemerin in food consumption and energy expenditure. We found that CMKLR1-deficient mice had markedly lower food consumption compared to wildtype controls. This suggests that chemerin/CMKLR1 may be a direct or indirect regulator of appetite, thereby establishing

that chemerin is a novel orexigenic adipokine. Future studies quantifying chemerin levels in the cerebrospinal fluid (CSF), determining which chemerin derivatives are present in the CSF, and measuring the effect of acute and chronic intrathecal chemerin treatment on food intake are required to identify mechanisms by which chemerin/CMKLR1 signaling mediates its orexigenic effect.

Adipokines have important autocrine/paracrine roles in regulating adipocyte differentiation and metabolism, and local inflammatory responses [163, 313-315]. TNFα is a pro-inflammatory adipokine expressed primarily in the SVF that inhibits adipogenesis and lipogenesis, and increases lipolysis [16, 200, 201]. In contrast, chemerin expression and secretion increases dramatically with adipogenesis, and loss of chemerin or CMKLR1 expression in preadipocytes severely impairs differentiation into mature adipocytes [228, 265]. Consistent with these *in vitro* findings, CMKLR1 loss *in vivo* resulted in a lower total body mass, primarily due to a significantly lower fat mass. Unfortunately, interpretation of this data is confounded by observed differences in food intake, since differences in body and fat mass could be solely the result of differences in feeding behavior. Indirect calorimetry and paired feeding suggests an altered metabolic phenotype, but based on our studies we can only conclude that the reduced body weight and adiposity associated with CMKLR1 loss primarily derives from differences in feeding and possibly, to some extent, from reduced adipogenesis. In the future, adipose tissue-selective CMKLR1 -/- mice would limit potential confounding factors, such as differences in appetite, and aid in more specifically determining the effect of CMKLR1 loss on adipogenesis *in vivo*.

Obesity is commonly associated with chronic low-grade systemic inflammation. This increase in inflammation commonly results in levels of pro-inflammatory/pro-

diabetic adipokines increasing, and levels of anti-inflammatory/anti-diabetic adipokines decreasing with obesity [16]. This increase in inflammation results in an increase in pro-inflammatory cytokine levels, which can interfere with numerous cellular processes, including insulin signaling [256]. For example, the serum concentration of the pro-inflammatory adipokine TNFα correlates with obesity, insulin resistance, fasting plasma glucose, insulin, and triacylglyceride concentrations [193, 198, 199]. In addition, TNFα promotes systemic insulin resistance by increasing fatty acid metabolites in muscle and/or liver tissue [200, 201]. IL-6 expression and secretion from adipose tissue also increases with obesity and induces insulin resistance in SKM, WAT, and liver [193]. Chemerin has been shown to promote chemotaxis of CMKLR1 expressing immune cells such as immature dendritic cells and macrophages [225]. CMKLR1 is now known to be expressed in a number of immune cells including plasmacytoid DCs, myeloid DCs, macrophages and natural killer cells [220, 226, 240]. Therefore, chemerin has the potential to influence numerous types of immune cells and thus contribute to obesity-associated inflammation. Prior to this work, there was no empirical evidence linking chemerin/CMKLR1 signaling with inflammation in adipose tissue or obesity. We have shown that CMKLR1 -/- mice have significantly lower pro-inflammatory cytokine mRNA levels in liver and WAT, significantly lower serum IL-6 levels, and a decrease in liver DC infiltration consistent with a pro-inflammatory role for chemerin/CMKLR1 signaling. Further analysis showed significantly less CD3+ T cells, and a significant increase in NK cells in WAT of CMKLR1 -/- mice. T cells are known to to mediate inflammation, proliferation, and production of the immune response. However, since different CD3+ T cell subsets have different roles in inflammation, future studies

measuring specific T cell subsets are required to determine the effect of this decrease on WAT inflammation. While few studies have reported the role of natural killer cells in WAT, one study demonstrated that the infiltration of natural killer cells into WAT is elevated in mice lacking mature T or B cells when fed a HF diet [307]. Since we found a decrease in CD3+ T cells, the increase in natural killer cells may represent a compensatory response of natural killer cells when T cells are lacking. While no other studies have investigated the role of natural killer cells in WAT inflammation and metabolism, one other study has shown that mice lacking natural killer T cells have improved glucose tolerance and decreased WAT macrophage infiltration [316]. The increase in natural killer cells found in CMKLR1 -/- WAT may have a similar effect in our model since we demonstrated exacerbated glucose intolerance in CMKLR1 -/- mice. Clearly, future studies are required to determine the role of natural killer cells in WAT. Taken together, our data suggests that CMKLR1 influences immune cell infiltration in liver and WAT. However, interpretation of this data is confounded by differences in metabolic phenotype. Since we found that CMKLR1-/- mice have a significantly lower body and fat mass compared to wildtype mice, differences in inflammation may be the result of a less obese phenotype in these mice. A future study using fat-selective chemerin knockout mice would be valuable in determining the role of chemerin/CMKLR1 signaling in WAT inflammation. Since chemerin is a pro-inflammatory adipokine that promotes chemotaxis of immune cells, we would expect chemerin-deficient WAT to have significantly less immune cell infiltration. This would result in a decrease in the expression of adipokines primarily derived from the SVF, such as TNFα, and an improvement in adiposity and insulin resistance. Studies have clearly shown that chemerin/CMKLR1 signaling influences immune cell infiltration and activation, and our

work suggests that obesity associated inflammation is also modulated by chemerin/CMKLR1 signaling. However, future studies are required to elucidate the specific function of chemerin in obesity-associated inflammation.

Several adipokines and their receptors have been implicated in adiposity and glucose homeostasis. For example, as previously mentioned, adiponectin regulates glucose metabolism by binding to two different receptors termed AdipoR1 and AdipoR2. AdipoR1 -/- mice show increased adiposity and decreased glucose tolerance, while AdipoR2 -/- mice are resistant to diet-induced obesity, and have an improved glucose tolerance [317]. Leptin also modulates glucose homeostasis; leptin-receptor deficient mice become identifiably obese, exhibit insulin resistance, glucose intolerance, and elevated plasma insulin and blood glucose levels [318, 319]. The elevated serum chemerin levels observed in humans and mice suggest that chemerin may also influence the dysregulation of glucose metabolism that often occurs with obesity. *In vitro* studies using 3T3-L1 adipocytes have provided conflicting results, with one study reporting decreased insulin-stimulated glucose uptake [291] and another showing increased insulin-stimulated glucose uptake and IRS1 tyrosine phosphorylation following chemerin treatment [282]. A studying measuring skeletal muscle cell glucose uptake showed that chemerin treatment increased phosphorylation of an IRS1 serine residue known for negatively modulating the actions of insulin, decreased Akt phosphorlyation, and decreased insulin-stimulated glucose uptake [278]. An independent study showed that overexpressing chemerin in mice caused glucose intolerance by inducing skeletal muscle insulin resistance [292]. Consistent with these studies, we have shown that treatment with recombinant chemerin exacerbated glucose intolerance in obese/diabetic but not normo-glycemic C57BL/6 mouse models, by decreasing serum insulin levels, and glucose uptake

in liver and WAT. In this study, given that the greatest impact of chemerin was on hepatic glucose uptake and serum insulin levels, modulation of GLUT2 and/or insulin secretion may be responsible for the observed differences. GLUT2 is a facilitative glucose transporter found primarily in the liver and pancreatic β-cells. In the liver, GLUT2 facilitates glucose uptake into liver, where it is then phosphorylated and used in various metabolic pathways. In β-cells, GLUT2 contributes to the regulation of insulin secretion by increasing the concentration of glucose within the cell. In obese/diabetic mice we observed that chemerin treatment during a glucose tolerance test caused a decrease in liver glucose uptake and serum insulin levels, suggesting that chemerin is reducing GLUT2 mediated glucose transport in these tissues. We have also shown that CMKLR1 loss results in a decrease in glucose-stimulated insulin secretion and a decrease in insulin-stimulated glucose uptake in skeletal muscle and WAT. However, the loss of CMKLR1 did not alter the insulin-evoked decline in blood glucose levels during an insulin sensitivity test suggesting that chemerin/CMKLR1 signaling did not reduce insulin sensitivity under these conditions. However, similar to current literature, these results are conflicting. *In vitro* studies using 3T3-L1 adipocytes have provided conflicting results and suggest that treatment dose and duration may determine if chemerin causes an increase or decrease in glucose uptake [282, 291]. For example, treating with 6 nM of chemerin for 12 h caused an increase in glucose uptake, treating with 10 μM of chemerin for 49 h resulted in a decrease in glucose uptake. In another study, treatment of primary human skeletal muscle cells with 60 nM chemerin for 24 h resulted in a decrease in glucose uptake [278], and overexpression of chemerin has been shown to exacerbate glucose intolerance by reducing glucose uptake in skeletal muscle [292]. Thus, the focus

of currently published literature has been the effect of chemerin on glucose uptake and the majority of data suggests that chemerin treatment inhibits glucose uptake. Similarly, results from our initial study suggest that chemerin treatment in obese/diabetic mice reduces glucose uptake by negatively modulating insulin secretion. However, data from CMKLR1 -/- mice suggests that CMKLR1 positively modulates insulin secretion. Intriguingly, chemerin treatment in lean wildtype mice had no effect on glucose uptake, while the largest difference in glucose tolerance in CMKLR1 -/- mice was found in LF fed mice of similar age, suggesting that the observed differences in insulin secretion are caused by different mechanisms. In addition, liver glucose uptake was unaffected in CMKLR1 -/- mice, but skeletal muscle and WAT glucose uptake was impaired. Therefore, while GLUT2 may be responsible for the observed differences in glucose uptake in chemerin treated obese mice, it is unlikely that it is causing the differences in CMKLR1 -/- mice since liver glucose uptake, which is mediated by GLUT2, is unaffected. Studies have also shown that both CMKLR1 and insulin secretion signaling result in intracellular calcium release, and phosphorylation of ERK kinases [47-49, 230, 308]. Therefore, the loss of CMKLR1 could impair insulin secretion by decreasing ERK activation and intracellular Ca^{2+} concentration resulting in a decrease in ATP-sensitive potassium channel activity and exocytosis of insulin-containing vesicles. Furthermore, differences in insulin secretion in CMKLR1 -/- mice may be due to developmental changes caused by CMKLR1-deficiency or differences in the overt metabolic phenotype found in these mice. Future studies specifically measuring glucose-stimulated insulin secretion from β cells in conjunction with chemerin treatment and/or CMKLR1 knockdown are required to determine the role of chemerin/CMKLR1 signaling in modulating the insulin secretion cascade.

In summary, we provide evidence that secretion of the novel adipokine chemerin is dynamic and modifiable with serum levels commonly increasing with obesity. Loss of the chemerin receptor CMKLR1 causes a decrease in appetite, adiposity, and energy consumption. Our data also demonstrates that CMKLR1 influences liver and WAT inflammatory responses that may be related to overall metabolic phenotype. Lastly, chemerin treatment and CMKLR1 loss worsened glucose tolerance due to decreased glucose stimulated insulin secretion and decreased liver, skeletal muscle, and WAT glucose uptake. Thus, we have shown that chemerin, similar to other adipokines, regulates a number of biological functions and may contribute to the pathology of obesity and obesity-related diseases, such as insulin resistance and type II diabetes, through the regulation and modulation of appetite, energy balance, adiposity, glucose homeostasis, and inflammation (Figure 28). However, a number of key research questions merit further investigation (Box 1). In particular, studies investigating which processed forms of chemerin are present in different tissues and the signaling function of the different processed forms on the chemerin receptors CMKLR1, GPR1, and CCRL2. In addition, the effect of WAT selective CMKLR1-deficiency on appetite, adiposity, and inflammation would provide critical insight into the role of chemerin in adipose tissue function with obesity. In conclusion, characterization of the function of chemerin and CMKLR1 in satiety, weight gain, and glucose tolerance has the potential to lead to novel therapeutic approaches for the treatment of obesity and type II diabetes.

Figure 28. The Novel Adipokine Chemerin Regulates Appetite, Energy Balance, Adiposity, Glucose Homeostasis, and Inflammation. The secretion of chemerin from white adipose tissue (WAT) is elevated in obesity. Chemerin from WAT promotes adipocyte differentiation, and alters adipocyte function. Chemerin also alters immune cell infiltration, modulates appetite and energy homeostasis, and has systemic effects on glucose homeostasis by altering pancreatic β-cell insulin secretion and glucose uptake in liver, skeletal muscle (SKM), and WAT. T = CD3+ T cell, NK = natural killer cell, DC = dendritic cell, CNS = central nervous system.

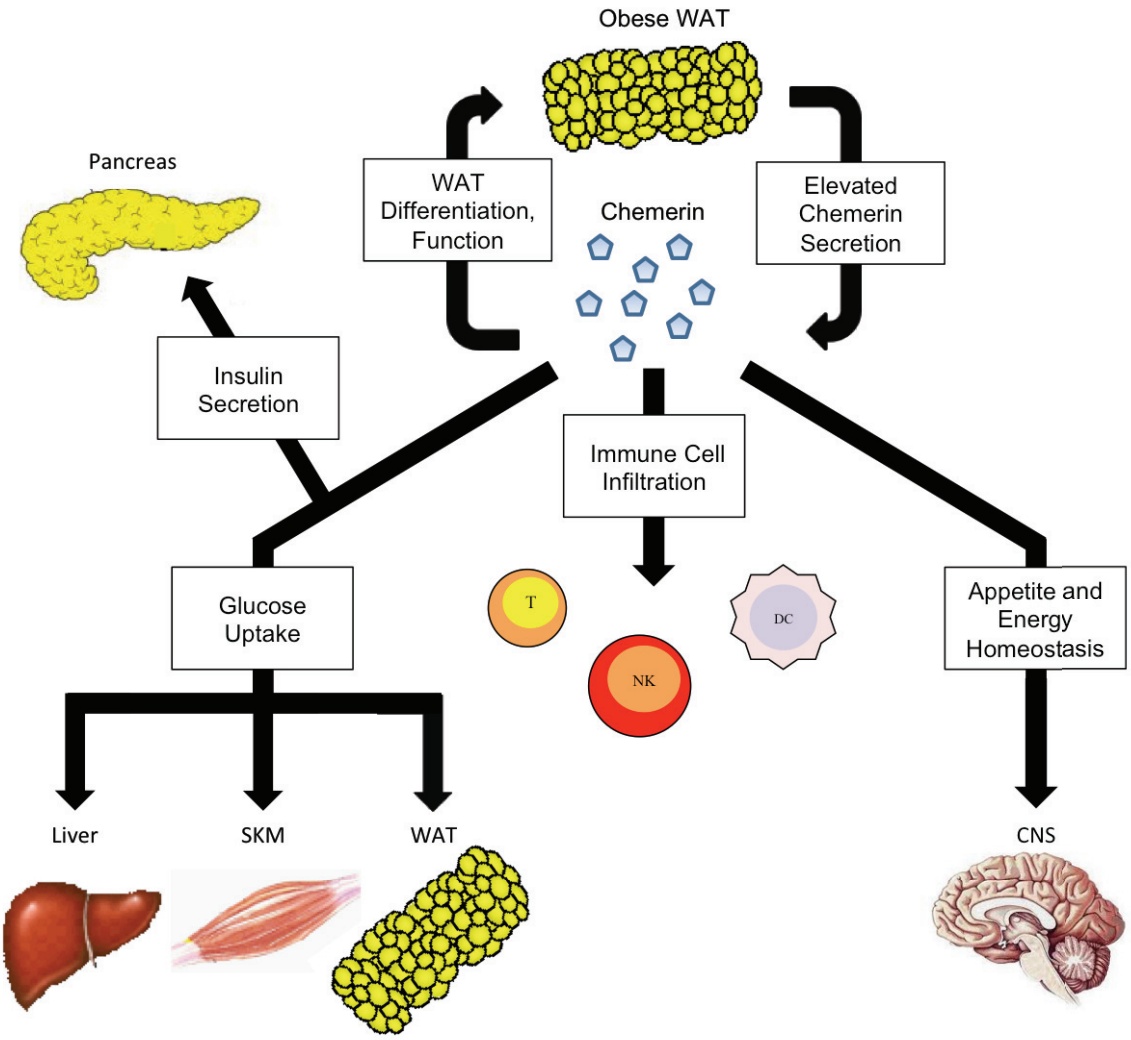

Obese WAT
Pancreas
WAT Differentiation, Function
Chemerin
Elevated Chemerin Secretion
Insulin Secretion
Immune Cell Infiltration
Glucose Uptake
T
DC
NK
Appetite and Energy Homeostasis
Liver
SKM
WAT
CNS

Table 5. Outstanding Research Questions.

1. Which processed forms of chemerin are present in different tissues and sites of tissue injury/inflammation?

2. What is the function of the chemerin receptors CCRL2 and GPR1?

3. What are the intracellular signaling pathways linked to chemerin activation of CMKLR1 and GPR1?

4. What is the expression profile of CMKLR1, GPR1, and CCRL2, and how is it affected by obesity?

5. Do the different chemerin receptors have different affinities for the various processed forms of chemerin, and how does the signaling function differ between the different forms?

6. Which processed forms of chemerin are found in the CSF, and how does chemerin/CMKLR1 signaling regulate appetite?

7. How does WAT-selective CMKLR1-deficiency affect adiposity, energy homeostasis, and inflammation?

8. Does WAT-selective chemerin/CMKLR1 signaling modulate inflammation in WAT?

9. How does chemerin/CMKLR1 signaling impact insulin secretion from pancreatic β-cells?

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Ernst, M.C., Haidl, I.D., Zúñiga, L.A., Zabel, B.A., Butcher, E.C. & Sinal, C.J. Targeted Disruption of the Chemokine Like Receptor-1 (CMKLR1) Gene is Associated with Reduced Adiposity and Glucose Intolerance. *Endocrinology*. Submitted July 2011.