Abstract

Therapeutic benefits of caloric restriction (CR) on clinical outcomes in the treatment of neurodegenerative disease, cancer, cardiovascular disease, and diabetes have been found. Studies indicate the positive health outcomes produced by CR may involve cellular nutrient-sensing pathways including insulin/insulin-like growth factor 1 signalling. CR has been reported to have significant effects on glucose metabolism and body composition: lowering fasting blood glucose and insulin, improving glucose tolerance, insulin sensitivity and decreasing energy expenditure and body fat. However, it is not clear which, if any, of the effects of CR are due to the lowering of circulating insulin. To determine which CR effects are a function of circulating insulin-dependent mechanisms we placed female $\text{Ins}1^{+/\cdot}$:$\text{Ins}2^{-/\cdot}$ mice and $\text{Ins}1^{+/\cdot}$:$\text{Ins}2^{-/\cdot}$ littermate controls on either a chow diet ad libitum (AL) or on a CR diet where they were fed 60% of what their genotype-matched littermate controls ate daily. All mice were singly housed and CR mice fed at night. With the onset of CR, body mass in both genotypes fell and reached a new equilibrium by 20 weeks of age. As expected, mice on CR had lower fasting, fed plasma glucose and improved glucose tolerance when compared to AL controls. We observed a more rapid return to baseline glucose post-insulin injection in mice on CR and no difference in glucose-stimulated insulin secretion compared to AL littermates. CR was able to prevent an age-dependent decline in fasting insulin of $\text{Ins}1^{+/\cdot}$:$\text{Ins}2^{-/\cdot}$ mice. $\text{Ins}1^{+/\cdot}$:$\text{Ins}2^{-/\cdot}$ and $\text{Ins}1^{+/\cdot}$:$\text{Ins}2^{-/\cdot}$ on CR also exhibited increased plasma leptin, glucose-dependent insulino tropic peptide, subcutaneous white and intrascapular brown adipose tissue size compared to the AL controls. The
endocrine milieu created in these very low insulin mice appears to disrupt several well-established effects of CR on body composition, insulin and insulin sensitivity.
Preface

For the entirety of the studies reported in this thesis I performed the design, data acquisition and analysis, writing and submission. Nicole Templeman and Betty Hu provided technical training for in vivo studies. Dr. Majid Mojibian aided in training on Xponent software for analysis of metabolic hormone data. Farnaz Taghizadeh helped in gross tissue dissection. The metabolic cage experiment data were obtained with aid from the lab of Dr. Susanne Clee. The data presented in chapter 3 will be submitted to the journal Aging Cell.

Animal Care Certificates were obtained and approved for this research (A11-0390 and A14-0197) with yearly renewal from the University of British Columbia.
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AgRp</td>
<td>Agouti-related protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine trisphosphate</td>
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<tr>
<td>AUC</td>
<td>Area under curve</td>
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<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CR</td>
<td>Caloric restriction</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>Foxo</td>
<td>Forkhead box O</td>
</tr>
<tr>
<td>Gck</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>Gip</td>
<td>Glucose-dependent insulinotropic polypeptide</td>
</tr>
<tr>
<td>Glp1</td>
<td>Glucagon-like peptide 1</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>Gpr40</td>
<td>Free fatty acid receptor 1</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein-2</td>
</tr>
<tr>
<td>Igf</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>Ins</td>
<td>Insulin</td>
</tr>
<tr>
<td>Irs</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>K$_{ATP}$</td>
<td>ATP-sensitive potassium channels</td>
</tr>
<tr>
<td>Mc4</td>
<td>Melanocortin 4</td>
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</table>
Mek  MAPK kinase
mRNA  Messenger ribonucleic acid
Npy  Neuropeptide Y
Pi3k  Phosphatidylinositol-3-OH kinase
PIP3  Phosphatidylinositol (3,4,5) trisphosphate
Pka  Protein kinase A
Pomc  Proopiomelanocortin C
Raf-1  RAF proto-oncogene serine/threonine-protein kinase-1
Ras  Rat sarcoma
RER  Respiratory exchange ratio
Shc  SH2-containing protein
SREBP-1c  Sterol regulatory binding element-1c
T3  Triiodothyronine
Tor  Target of rapamycin
Ucp1  Uncoupling protein 1
VLDL  Very low density lipoprotein
VMH  Ventromedial hypothalamus
WAT  White adipose tissue
α-MSH  α-melanocyte stimulating hormone
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Of my seven years at UBC the last two have by far been the most exciting, challenging and rewarding. Throughout my undergraduate degree I wanted to be a part of the scientific community and make my own contribution to medical research. I am so thankful to my supervisor James D. Johnson for giving me the opportunity to fully embrace my intrigues and interests in the lab. His support and guidance throughout my research have been irreplaceable.

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To my best friends Alisa, Mai, Christina and David, you’ve all helped keep me sane and maintain my motivation throughout my many years at UBC. To my family and partner Joey, your never-ending love and support have always been such a wondrous foundation. It has allowed me to succeed and progress in ways I could never have done without you.
Dedication

This thesis is dedicated to my friends, family and partner.
Chapter 1: Introduction

1.1 Insulin function and regulation

1.1.1 Diabetes and the discovery of insulin

Diabetes mellitus is a disease characterized by an inability to produce sufficient insulin hormone and it results in continual elevated levels of blood glucose. Two types of diabetes mellitus exist. Type 1 diabetes arises as a result of autoimmune destruction of the insulin producing cells (~5-10% of cases), requiring patients to undergo lifelong insulin therapy. Type 2 diabetes mellitus (>90% of cases) is designated once production of insulin is insufficient to meet physiological demand\(^1\). The risk of developing type 2 diabetes increases as a function of and obesity and age\(^2\).

Insulin has been a lifesaving treatment for millions of people with diabetes worldwide since its discovery. The story of insulin discovery begins with Paul Langerhans in 1868 when he observed that pancreata from diabetic patients were damaged. Clusters of cells existed in the pancreas with unknown function, that would become known as the pancreatic islets of Langerhans\(^3\). Next, Oskar Minkowski and Joseph von Mering discovered that dogs became diabetic after removal of their pancreas but maintained euglycemia upon pancreatic duct ligation in 1889\(^4\). Several years later in 1920 with the aid of his student Charles Best and funding from John Macleod, Frederick G. Banting injected pancreas extract into pancreatectomized dogs and found that they were able to reverse the dogs' diabetic symptoms. After purifying the extracts with the aid of Bertram Collip, they began testing in humans. The first diabetic patient to be treated with
a purified version of the extract, Leonard Thompson, showed remarkable recovery from his diabetic symptoms and near-death state. The identification of insulin as the first critical hormone controlling glucose homeostasis is regarded as one of the most significant discoveries in medical history. Justifiably, Frederick G. Banting and John Macleod were awarded the Nobel Prize in Physiology or Medicine in 1923 for the discovery of insulin. Improvements and alterations on insulin therapy are still underway today and new roles of this hormone maintain an exciting research environment for those studying diabetes and related-diseases.

1.1.2 Insulin across phylogeny

Insulin, insulin-like growth factors (Igf) and their downstream effectors involved in nutrient sensing are conserved from worms to humans. Evolutionary conservation of a gene or signalling pathway generally implies a significant role in and necessity for survival. In *Caenorhabditis elegans* there are 40 insulin-like genes, in *Drosophila melanogaster* there are 7. In worms and flies, insulin-related hormones are produced in specialized neuroendocrine cell types. Specifically, in flies the insulin-like peptides are produced in neuroendocrine cells in the pars intercerebralis region of the protocerebrum as well as in the salivary gland and midgut. In both organisms they serve in sensing and responding to nutrients and in lifespan regulation. For instance, mutation of *daf-2*, encoding for the sole *C. elegans* ortholog of the insulin/Igf-1 receptor, lead to significantly increased lifespan. In *D. melanogaster*, ablation of cells producing insulin-like
peptides increased hemolymph glucose levels and lipid storage in the fat body, and maintained a higher resistance to oxidative stress, starvation and have increased lifespan\textsuperscript{11,16}. Similarly, in mammals, including humans, insulin and Igfs are involved in energy storage, metabolism, growth and lifespan\textsuperscript{7,17,18}. It is evident that insulin and related pathways have maintained common function and importance across phylogeny.

1.1.3 Regulation of mammalian insulin expression and secretion

In mammals, insulin is produced by the \( \beta \)-cells in the islets of Langerhans of the pancreas. Mammalian insulin genes encode for preprohormones and require post-translational modification by two prohormone convertase enzymes (Pc1 and Pc2) and exopreptidase carboxypeptidase E\textsuperscript{19}. Prohormone convertase 1 and 2 cleave at two sites located after pairs of basic residues (lysine-64 and arginine-65, and arginine-31 and arginine-32) releasing a peptide fragment, C-peptide\textsuperscript{20}. Once the C-peptide has been cleaved, the two pairs of basic residues are removed via carboxypeptidase E\textsuperscript{20}. Mature insulin consists then of two peptide chains (A and B), which are connected by two disulfide bonds and packaged in vesicles\textsuperscript{20}. Insulin granules are packaged as hexamer crystals complexed with zinc ions in intracellular vesicles ready for secretion\textsuperscript{21-23}.

The cellular production of insulin is regulated at multiple processing levels which are primarily affected by glucose metabolism\textsuperscript{24}. Transcription of the human \textit{INS} and the rodent \textit{Ins2} genes are regulated by a 340-660 bp promoter region upstream of the transcriptional start site\textsuperscript{24-26}. This region is composed of binding
sites for ubiquitous as well as β-cell-specific transcription factors\textsuperscript{25,27}. It should be noted there are some differences in the promoter elements between the rodent \textit{Ins2} gene and the human \textit{INS} gene, including binding sites and regulatory element spacing\textsuperscript{28}. However, the main three regulatory elements (A3, E1 and C1) that are involved in the response to glucose are conserved\textsuperscript{28}. There are three main transcription factors that function in coordination with each other and synergistically increase insulin gene expression as a result of increasing glucose levels\textsuperscript{29-31}. These factors are Pdx1 (pancreatic and duodenal homeobox-1), NeuroD1 (neurogenic differentiation 1), and MafA (\textit{V}-maf musculoaponeurotic fibrosarcoma oncogene homologue A)\textsuperscript{32-43}. Glucose affects the transcriptional regulatory activity of these factors by different mechanisms. Glucose alters Pdx1 localization, DNA-binding affinity and interaction with other proteins\textsuperscript{44-50}. NeuroD1 translocates to the nucleus and MafA transcription increases dramatically upon β-cell exposure to increased glucose concentration\textsuperscript{51-56}.

Insulin production can be regulated by hormones. Insulin may also function in an autocrine or paracrine fashion on β-cells in a positive feed-forward mechanism that has shown to increase insulin synthesis in dispersed human islet cells\textsuperscript{57}. Leptin, an adipose tissue secreted hormone, also reduces insulin gene transcription\textsuperscript{58,59}. Growth hormone can also affect insulin transcription. Rats with a growth hormone producing tumor had increased ratio of \textit{Ins1} to \textit{Ins2} expression, suggesting the two genes are regulated differently\textsuperscript{60}. Additionally, glucagon-like peptide, a gut secreted incretin hormone, promotes glucose
regulation of insulin gene transcription via increasing Pdx1 levels and activity\(^{61-64}\).

Insulin expression is also regulated post-transcriptionally by rate of translation and mRNA degradation by interactions with RNA-binding proteins and microRNAs\(^{65}\). RNA-binding proteins that specifically enhance insulin mRNA stability include the Hu family of proteins and polypyrimidine tract-binding protein\(^{65}\). Insulin mRNA represent at least 30% of rat islet cell mRNA; insulin mRNA has a half-life ranging from 29 to 77 hours depending on glucose concentrations\(^{66,67}\). Rapid increases in insulin production due to rising glucose levels are a result of increases in insulin mRNA translation, whereas transcriptional processes occur more slowly\(^{68-70}\).

Insulin secretion is regulated by circulating blood glucose levels, but can also be induced by other circulating nutrients such as fatty or amino acids\(^{71,72}\). The mechanisms by which nutrients cause insulin secretion are well studied. Glucose passes into the \(\beta\)-cell from the blood via the high capacity transmembrane glucose transporters. GLUT1 is the primary transporter in humans, whereas Glut2 is the primary transporter in rodents\(^{73}\). Once inside the \(\beta\)-cell glucose is rapidly phosphorylated by glucokinase whereupon the phosphorylated glucose-6-phosphate enters glycolysis. This leads to increases in intracellular adenosine-triphosphate (ATP) and a shift in the ATP:ADP ratio causing closing of the ATP-sensitive potassium channels (\(K_{\text{ATP}}\)), cell depolarization and entry of \(\text{Ca}^{2+}\) through voltage-gated calcium channels leading to fusion of insulin-containing vesicles with the cell membrane and thus
secretion\textsuperscript{73,74}. Circulating fatty acids can bind the fatty acid receptor Gpr40, which ultimately leads to an increase in intracellular Ca\textsuperscript{2+} and insulin secretion\textsuperscript{72}. Fatty acids are also able to amplify glucose-stimulated insulin secretion via intracellular metabolism of fatty acids causing increased ATP production\textsuperscript{72}. Insulin secretion is tightly regulated and is affected by other hormones, such as the incretins and potentially the novel decretins secreted by enteroendocrine cells of the gut\textsuperscript{75,76}. The incretins, glucagon-like peptide 1 (Glp1) and glucose-dependent insulinotropic polypeptide (Gip) produced by L and K cells, respectively, function to potentiate post-prandial insulin secretion. Both incretins act by binding to their respective G-protein coupled receptors on β-cells causing increases in intracellular cyclic-AMP levels and downstream effects that amplify insulin secretion in a glucose-dependent manner\textsuperscript{76}. The novel decretin, Neuromedin U, has recently been shown to have the opposing effect and decreases insulin secretion in human islet β-cells\textsuperscript{75}. However, more \textit{in vivo} data is required to determine its physiological relevance. Leptin also acts to decrease insulin secretion via several mechanisms\textsuperscript{77-81}. Insulin is a vital nutrient-sensing hormone and β-cells integrate many signalling pathways to supply appropriate insulin levels dependent on nutritional and endocrine environment.

\subsection{Murine \textit{Ins1} and \textit{Ins2} expression pattern}

Unlike humans who have one \textit{INS} gene, mice and rats have two, the \textit{Ins1} and \textit{Ins2} genes\textsuperscript{82-84}. They are thought to be the result of a gene duplication event by retroposition of partially processed \textit{Ins2} mRNA\textsuperscript{82}. The peptides that
arise from these genes are not currently believed to have different biological activity or receptor affinity. The murine Ins2 gene has greater homology to the human INS than Ins1 and is regarded as the more ancestral gene. The Ins1 gene is thought to have near exclusive expression in the pancreas, whereas Ins2 is expressed in the pancreas, brain and thymus. As the thymus is involved in eliminating auto-reactive T-cells, mice lacking Ins2 expression in this organ might be expected to be more susceptible to autoimmune destruction of β-cells.

Indeed, non-autoimmune, early onset insulin-deficient diabetes has been observed in male Ins1+/−:Ins2−/− mice on the nonobese diabetes mouse background. In female Ins1+/+:Ins2−/− or Ins1+/−:Ins2−/− on the same background also develop insulin-deficient diabetes accompanied by insulitis. However on a 129/Sv background mice lacking either the Ins1 or Ins2 gene are able to compensate and maintain similar circulating insulin levels, euglycemia and do not become diabetic. Mice completely lacking both Ins1 and Ins2 develop neonatal diabetes and cannot survive without insulin treatment. Hypoglycemia was not a common phenotype of Ins2−/− mice in a recent study in our lab with mice obtained from the Bucchini group and partially backcrossed with C57BL/6 mice. In this study Ins1 and Ins2 expression profiles in the central nervous system of these mice was determined in detail. The highest expression level of Ins2 in postnatal mice was in the hippocampus, minimal expression of Ins1 was observed only in spinal cord, medulla and olfactory bulb. This suggests murine neural insulin production is almost exclusively from the Ins2 gene. Whole-body tissue Ins1 expression profile data was also summarized revealing expression is
primarily in the pancreas with minimal expression in placenta, lung and urogenital sinus\textsuperscript{85}. Thus although the peptides produced from \textit{Ins1} and \textit{Ins2} are nearly identical, the regulation of their expression likely differ due to differences in regulatory elements upstream of the transcription start site\textsuperscript{91}.

1.1.4 Insulin signalling

Insulin has functions in many cellular and physiological processes including cell growth and survival, energy homeostasis, glucose metabolism, feeding, learning and lifespan\textsuperscript{7,17,18}. The insulin protein functions in an autocrine, paracrine and endocrine fashion by binding receptors expressed by peripheral and central tissues. The primary receptor for insulin is the insulin receptor, although insulin may also bind hybrid insulin/lgf1 receptors\textsuperscript{18}. Upon binding to and activating the insulin receptor, its tyrosine kinase activity causes autophosphorylation at various tyrosine residues on the intracellular side of the receptor. The phosphorylated sites then attract and interact with signal amplifiers and effectors like the insulin receptor substrate proteins, including Irs1 and Irs2, as well as Shc (SH2-containing protein)\textsuperscript{18}. The ultimate effects of insulin receptor activation depend on the cell type and which signalling effectors are expressed. One of the primary pathways involve activation of Grb2 (growth factor receptor-bound protein-2), Ras (rat sarcoma), Raf-1 (RAF proto-oncogene serine/threonine-protein kinase-1), Mek (MAPK kinase), and finally result in phosphorylation of Erk1 and Erk2 (extracellular regulated kinases) causing translocation to the nucleus where they can alter gene expression\textsuperscript{18}. The second
pathway requires activation of phosphatidylinositol-3-OH kinase (Pi3k), production of phosphatidylinositol (3,4,5) trisphosphate (PIP3), activating Akt which can cause translocation of Glut4, a glucose transporter, to the membrane increasing cell permeability to glucose, as well as phosphorylate the transcription factor forkhead box O (Foxo) leading to its translocation out of the nucleus thereby changing gene expression. The signals briefly described here do not exhaustively describe all the pathways and effector proteins that insulin signalling interacts with and affects. The cellular outcomes as a result of insulin receptor binding depend strongly upon the presence of endocrine or other signals being received by the cell, the cell type and its expression profile.

### 1.1.5 Physiological effects of insulin on peripheral tissues

In most tissues insulin is primarily involved in increasing cell membrane permeability to glucose via translocation of Glut4 to the plasma membrane but it has many other anabolic effects that are tissue-dependent. In the liver, insulin promotes glycogen, protein and fatty acid production from glucose and inhibits gluconeogenesis and glycogenolysis. In white adipose tissue (WAT), insulin signalling causes increased fatty acid synthase activity, storage of triglycerides and inhibits lipolysis thereby increasing lipid energy stores. In skeletal muscle, where the majority of acute insulin-dependent glucose uptake is thought to occur, there is also a mild increase in glycogen production and storage.

The pathology of diabetes includes the development of insulin resistance, which results in dysregulation of glucose and lipid metabolism. This occurs
when tissues that are normally sensitive to insulin become unresponsive to physiological levels of insulin. This may be the cause or consequence of increased circulating insulin levels, hyperinsulinemia, that occurs in the development of type 2 diabetes mellitus and remains under debate. Our lab recently reported that the genetic prevention of hyperinsulinemia in male \( \text{Ins1}^{+/}\) :\( \text{Ins2}^{-/-} \) mice prevents development of insulin resistance despite being on a high fat diet for a year and having different body weights.

The importance of insulin signalling for maintenance of healthy physiology are evinced by the phenotypes of tissue-specific insulin receptor knockout mice from key insulin responsive tissues. Muscle-specific insulin receptor knockout mice maintain normal glucose tolerance, insulin sensitivity and insulin levels with mild dyslipidemia. The liver-specific deletion of insulin receptor causes insulin resistance, hyperinsulinemia, glucose intolerance and hyperglycemia in mice. Mice with β-cell specific knockout of the insulin receptor exhibit decreased insulin secretion in response to glucose challenge, develop impaired glucose tolerance and hyperinsulinemia. The phenotype of fat specific insulin receptor knockout mice include increased lifespan, protection from age-related glucose intolerance, decreased fat mass and disruption of the leptin levels that are normally positively correlate with fat mass. When the insulin receptor is knocked out of the mouse brain mice have increased body weight, WAT mass, increased food intake in females despite increased leptin. These mice also developed insulin resistance, hypertriglyceridemia and increased circulating insulin in females. The physiological effects of insulin are many and differ by tissue but it is evident
glucose and lipid homeostasis as well as body weight are important functions of this anabolic hormone\textsuperscript{18,100}.

**1.2 Physiological regulation of glucose levels**

Maintenance of physiological levels of blood glucose are extremely vital as our brains are only able to metabolize two carbon fuels: glucose and ketones\textsuperscript{101}. Untreated hypoglycemia, the lack of sufficient blood glucose, can lead to coma and death. Hypoglycemia can occur during starvation or in people with diabetes as a result of incorrect insulin dosing\textsuperscript{102}. On the other end of the spectrum, having consistent high blood glucose, or hyperglycemia, as is seen in diabetes patients not maintaining proper glycemic control, has several pathological effects. These include various micro and macrovascular complications such as neuropathies, retinopathies and ulcers. In order to prevent the detrimental effects of glycemic dysregulation and pathological levels of circulating glucose, mammals have evolved several biological systems that function to maintain euglycemia.

The main tissues involved in glycemic control are the islets of Langerhans of the pancreas, the liver, adipose tissue, skeletal muscle, the brain, the kidneys and adrenal glands\textsuperscript{92,101}. Each tissue plays a specific role in the system. The $\beta$-cells and $\alpha$-cells found in the islets of Langerhans secrete insulin and glucagon, respectively, with opposing function in terms of effects on blood glucose. Insulin is an anabolic hormone and acts to decrease blood glucose by causing translocation of Glut4 from vesicles to the plasma membrane causing increased
tissue uptake of glucose, whereas glucagon acts on the liver to increase circulating glucose levels. The liver responds to circulating hormones by increasing or decreasing blood glucose appropriately. In order to increase blood glucose hepatocytes begin to break down glycogen and fat stores and initiate gluconeogenesis. The opposite occurs when circulating glucose is sufficient, resulting in glycogen and fat storage and inhibition of gluconeogenesis.\textsuperscript{92,101} Skeletal muscle is the tissue responsible for the most insulin-dependent glucose uptake and myocytes are also able to store minimal amounts of glycogen for fuel use during fasting or exercise.\textsuperscript{101} The brain has hormone and nutrient integrating centers that are able to respond to endocrine stimuli and exert top-down neuroendocrine control on glucose and energy homeostasis (discussed further later). The kidneys are able to produce glucose via gluconeogenesis and are involved in glucose recycling or in cases of hyperglycemia excess glucose is secreted and eliminated through urination.\textsuperscript{103} The adrenal glands function in glucose regulation by secreting cortisol (or corticosterone in rodents) by zona fasciculata-reticularis and adrenaline by chromaffin cells of the adrenal medulla in response to stress-stimuli through the neuroendocrine axis.\textsuperscript{104,105} The acute stress hormone adrenaline has many physiological effects. With regards to energy homeostasis, adrenaline causes lipolysis in adipose tissue, glycogenolysis in liver and gluconeogenesis in the kidneys in order to increase circulating glucose.\textsuperscript{104}
Adipose tissue affects glucose homeostasis through both endocrine and non-endocrine mechanisms\textsuperscript{106}. Adipocytes secrete many proteins with endocrine action, named adipokines, and with roles in glucose homeostasis. The first such protein discovered, leptin, has many actions. With regards to glycemia, leptin improves both muscle and liver insulin sensitivity, corrects hyperglycemia and hyperinsulinemia in leptin deficient \textit{ob/ob} mice\textsuperscript{58,107-109}. Leptin plays a significant role in maintaining whole-body insulin sensitivity, insulin controlled liver glucose production and suppresses insulin secretion from β-cells\textsuperscript{110}. These effects are independent of leptin’s effects on food intake\textsuperscript{81}. Leptin has been shown to decrease levels of counterregulatory hormones, which may be part of the mechanism by which leptin ameliorates glucose homeostasis\textsuperscript{81,109,111-113}. Another important adipokine that also increases insulin sensitivity is adiponectin\textsuperscript{114}. Adiponectin’s effects on insulin sensitivity and improved glucose homeostasis may be a combination of β-cell protection from apoptosis, increased Glut4 translocation in muscle, suppression of hepatic glucose output and its anti-inflammatory properties\textsuperscript{114-117}. Other adipokines with positive effects on glucose homeostasis include omentin and visfatin, while still others, tumor necrosis factor-α, resistin and retinol-binding protein 4 have negative effects on insulin sensitivity and glucose homeostasis\textsuperscript{106}. Adipocytes secrete non-esterified fatty acids, especially during fasting, which decrease muscle glucose uptake and increase hepatic glucose production\textsuperscript{118,119}. Acutely, non-esterified fatty acids induce insulin secretion in the post-prandial state and sensitize β-cells to glucose\textsuperscript{106,120}. However, chronically high circulating fatty acids decrease insulin
secretion and can cause β-cell death\textsuperscript{120-122}. A final function of adipose tissue in glucose homeostasis involves it serving as a safe lipid storage site. Ectopic lipid accumulation in the liver or muscle increase insulin resistance in these tissues and exacerbate diabetes\textsuperscript{106,122,123}. Dysregulation of adipose tissue and its endocrine functions have a significant impact on glucose homeostasis and provide a major link between obesity and the development of type 2 diabetes. Unsurprisingly, many tissues are involved in the proper maintenance of circulating glucose as it is so vital to maintain physiological glucose levels for health and survival. Many therapeutics are targeted to various components of glucose regulation for the treatment of diabetes.

1.3 Regulation of body weight and energy homeostasis

1.3.1 Regulation of body weight by insulin

Another major function of insulin is in long-term body weight management and energy expenditure. The mechanisms regulating body weight are being extensively studied. The discovery of potential methods for the treatment and prevention of obesity, a growing epidemic, is of health and commercial interest. Despite significant variations in day-to-day food intake body weight is maintained within a small range, which may be a reason why treating obesity is so difficult\textsuperscript{124}. This long-term stability is provided by mechanisms of energy balance that alter energy expenditure dependent on energy intake\textsuperscript{125,126}. Research has established that changes in body weight are defended and that a set-point is a function of both environmental and genetic factors\textsuperscript{127}. Body weight defense
suggests integration of peripheral and central signals that act to alter metabolic activity to account for variations in energy intake\textsuperscript{124}. One of these signals is insulin. This hormone is well known for its acute anabolic effects on body weight and fat mass. Indeed, post-prandial and fasting insulin correlate with fat stores\textsuperscript{128,129}. Part of insulin’s body weight regulation role, involves effects on feeding behaviour. This is suggested by the decreased feeding after intracerebroventricular administration of insulin to streptozotocin-induced diabetic rats\textsuperscript{130}. Insulin’s inhibitory effects on feeding are evinced by the hyperphagia seen in female neuron-specific insulin receptor knockout mice\textsuperscript{99}. Even oral administration of an insulin mimetic, more permeable to central nervous system than insulin, decreased food intake, body weight and minimized weight gain in mice fed a high fat diet\textsuperscript{131}. Circulating insulin is known to cross the blood brain barrier by a saturable mechanism\textsuperscript{132,133}. The insulin receptor is expressed by neurons in the brain\textsuperscript{134}. However, circulating insulin isn’t the only source of insulin in the brain. As mentioned earlier hippocampal and olfactory bulb neurons express insulin\textsuperscript{85,135-138}. It is now well established that insulin exerts its effects on feeding and long-term body weight management via central effects on specialized neurons in the ventromedial hypothalamus\textsuperscript{139,140}. The mechanisms of which are discussed below. Perhaps the most convincing evidence is the neuron specific insulin receptor knockout mouse, which displays increased food intake and fat mass\textsuperscript{99}. Similarly, mice exhibited the same phenotype when injected with insulin receptor antisense oligonucleotides near the hypothalamus\textsuperscript{141}. Thus insulin plays a significant role in controlling body
weight by both acute peripheral and chronic central mechanisms. Although insulin role is important it is not the only hormone with effects on body weight regulation.

1.3.2 Leptin regulates body weight and energy expenditure

Another hormone with a significant role in energy store regulation and maintenance of energy expenditure at appropriate levels is a fat secreted hormone, leptin. One of the initial observations implicating fat in energy regulation was in rats, where food intake, indicative of an energy deficit, was affected by fat depot size\textsuperscript{142}. Later, two spontaneous mutations of either the \textit{ob} or \textit{db} genes were discovered, where mice homozygous for either mutated gene were phenotypically identical\textsuperscript{143}. These mice exhibited a three fold increase in body mass compared to normal mice on the same diet and five times the body fat content\textsuperscript{143}. The gene products of these genes were eventually identified as the hormone leptin and its cognate receptor\textsuperscript{144,145}. Leptin is now known to be a hormone secreted by adipose tissue, or adipokine, involved in food-intake, metabolism and fertility\textsuperscript{146,147}. Circulating levels of this hormone are correlated with WAT mass, decrease as a function of weight loss and are increased in obese humans and rodent models of obesity\textsuperscript{148,149}. Mutant \textit{ob/ob} mice exhibit similar pathophysiology to starved animals such as decreased body temperature and energy expenditure, hyperphagia and infertility\textsuperscript{143}. Treatment of these mice with leptin returns these parameters to physiological levels, suggesting a state of perceived starvation where the animal’s response is to store all ingested excess
nutrients resulting in obesity\textsuperscript{107,147,150}. Leptin functions to resist changes in weight in both directions and is a major regulator of long-term body weight\textsuperscript{151-153}. Leptin and other hormones act centrally in the hypothalamus to regulate energy expenditure. Energy expenditure can be categorized into basal metabolism, physical activity and adaptive thermogenesis. The specific neuroendocrine mechanism whereby insulin and leptin affect feeding behaviour, body weight and energy expenditure involve binding to receptors expressed on neurons in the ventromedial hypothalamus.

1.3.3 Neuroendocrine axis: feeding behavior and energy expenditure

One of the major organs integrating energy intake and modifying energy expenditure is the brain, the effector nuclei are the ventromedial and arcuate nuclei, which contain nutrient-sensing neurons and are located in the ventromedial hypothalamus \textsuperscript{146,154}. The primary function of these neural networks is controlling body weight, feeding and energy expenditure \textsuperscript{155,156}. These neuronal populations are sensitive to insulin and leptin \textsuperscript{157-159}. Insulin and leptin have similar effects on these neurons and act as satiety factors and lead to decreases in food intake \textsuperscript{157,158}. Leptin binds to its receptor (Lepr) on two types of neurons expressing either neuropeptide Y (Npy) and Agouti-related protein (Agrp) or proopiomelanocortin C (Pomc) and cocaine and amphetamine regulated transcript (Cart) in the arcuate nucleus \textsuperscript{160}. In the Pomc/Cart neurons leptin increases the production of Cart and α-melanocyte stimulating hormone (α-Msh), a melanocortin 4 (Mc4) agonist \textsuperscript{160}. Contrastingly leptin inhibits production of Npy
and Agrp, an antagonist of the Mc4 receptor\textsuperscript{160}. These signals are integrated by Mc4 receptor expressing neurons, which are involved in regulating metabolic rate, feeding, energy expenditure, insulin secretion and glucose homeostasis\textsuperscript{160,161}.

The mutation of the insulin receptor specifically in the brain causes hyperphagia, obesity, insulin resistance, increases in circulating triglycerides and leptin in female mice\textsuperscript{99}. In addition these neurons are sensitive to changes in physiological levels of glucose, above that required by the brain\textsuperscript{162-166}. They also control whole-body glucose homeostasis: demonstrated by the specific increase or decrease in ventromedial hypothalamus glucose metabolism preventing or inducing peripheral counterregulatory responses to hypoglycemia, respectively\textsuperscript{167-169}. In addition, leptin infusion into the ventromedial hypothalamus of rats results in increased peripheral glucose metabolism\textsuperscript{170}. While ablation of leptin receptors in the Agrp/Npy neurons of the arcuate nucleus causes obesity in mice due to temporary hyperphagia and decreased energy expenditure\textsuperscript{171}. A mechanism by which energy expenditure may be decreased is by altering thermogenesis. A process involving sympathetic nerve stimulation in the brown adipose tissue\textsuperscript{172,173}.

1.3.4 Brown adipose tissue in energy balance

Brown adipose tissue (BAT) is important for proper glucose homeostasis, energy balance and thermogenesis. BAT was previously believed to only exist in humans in early infants, however more recent positron-emission tomography
results found that there is metabolically active BAT in healthy adult humans$^{174}$. BAT is characterized by dense vasculature, multilocular intracellular lipid droplets and a high density of mitochondria$^{172}$. Brown unlike WAT is normally an energy burning tissue as opposed to an energy storing tissue$^{172,175}$. Mice lacking BAT become obese$^{175}$. BAT is implicated in glucose homeostasis as mice lacking insulin receptor in BAT develop glucose intolerance likely due to an insulin secretion defect but remain insulin sensitive$^{176}$. Additionally, mice with BAT transplants have increased insulin sensitivity and glucose tolerance$^{177}$. However, the primary function of BAT in rodents is thought to be thermoregulation by its defining ability to uncouple the proton gradient created by the electron transport chain of the inner mitochondrial membrane during oxidative phosphorylation of energy fuels$^{172}$. The protein responsible for this effect is the uncoupling protein 1 (Ucp1), which is uniquely highly expressed in BAT, but can be induced in WAT by sustained cold stress, β-adrenergic compounds or insulin$^{85,176,178}$. The expression of Ucp1 in BAT as well as BAT size can be increased by exposure to cold or a result of adrenergic stimulation of the β-adrenergic receptors$^{172}$.

It has recently become clear that cells within WAT can also adopt some features of BAT, albeit in a very limited capacity. The induction of Ucp1 expression during the ‘browning’ of WAT may be inhibited by insulin$^{85}$. Specifically, in male $\text{Ins1}^{+/-}:\text{Ins2}^{-/-}$ mice, with decreased circulating insulin, exhibit increased Ucp1 expression in WAT on high-fat diet compared to $\text{Ins1}^{+/-}:\text{Ins2}^{-/-}$ controls$^{85}$. This suggests insulin negatively regulates Ucp1 expression in WAT,
which agrees with the decreased Ucp1 expression observed in brown adipocyte specific insulin receptor null mice\textsuperscript{176}. Knowing there is active BAT in adult humans makes this tissue and conversion of WAT to brown-like or “beige” an attractive therapeutic target for the treatment of obesity\textsuperscript{175,179}.

1.4 Body composition and fat mass

1.4.1 Adipose tissue subtypes and function

Adipose tissue is the largest mammalian endocrine organ and is involved in controlling several biological functions through adipokine secretion including food intake, glucose homeostasis, insulin sensitivity, aging, fertility and body temperature\textsuperscript{180}. There are different WAT depots including subcutaneous, inguinal, interscapular, perigonadal, retroperitoneal and mesenteric depots\textsuperscript{180}. Adipose tissue found in the abdominal cavity (or visceral fat) has altered metabolic and functional roles from subcutaneous adipose tissue, having a more extensive capillary bed, sympathetic efferent innervation and has adipogenic, pro-atherogenic and pro-thrombotic properties\textsuperscript{181-183}. There are also differences in the secretory profile between WAT depots, for example leptin and adiponectin are secreted at higher levels from subcutaneous adipocytes, whereas retinol binding protein 4 and plasminogen activator inhibitor 1 are more selectively secreted by visceral adipose tissue\textsuperscript{184-187}.

There are sexual dimorphisms in fat depot size. Women tend to have increased total adipose size over lifespan as well as increased ratio of subcutaneous to visceral fat\textsuperscript{188}. These sex depot-specific patterns may be a
function of sex hormones; however, the exact mechanism for the dimorphisms have yet to be elucidated\textsuperscript{189-191}. A recent genome-wide association study suggests there may be a genetic component to sexual dimorphic body fat distribution. Surgical excision of visceral fat improves glucose homeostasis in both male and female mice, whereas removal of subcutaneous fat offers no improvements in rodent or human metabolic syndrome\textsuperscript{181,192-194}. Additional differences between the two types of WAT include their mechanisms for expansion during high-fat diet, where visceral fat primarily expands by hypertrophy, increase in adipocyte size, and subcutaneous fat through hyperplasia, recruitment of new adipocytes by replication and differentiation of adipocyte progenitor cells\textsuperscript{188,195}. Unsurprisingly, there are also differences in lipid metabolism in subcutaneous compared to visceral fat depots. Their varying metabolic and secretory profiles substantiate the more negative outcomes associated with increased visceral fat\textsuperscript{182,192,196-198}.

1.4.2 Fat storage plays a role in maintaining good health

Since the discovery of leptin, appreciation for the role of adipose tissue in maintaining healthy physiology has increased dramatically. The role of fat in maintaining health is made evident by the complications and comorbidities arising from either lipodystrophies, genetic or acquired disorders exhibiting selective loss of body fat, as well as those associated with excess body fat in obesity. Patients with partial or generalized lipodystrophy, where there is fat loss from many or all depots, are predisposed to hypertriglycerideridemia, hepatic
steatosis, polycystic ovaries, acanthosis nigricans, insulin resistance and thus type 2 diabetes mellitus\textsuperscript{199,200}. Some of the same health risks arise in obese patients such as insulin resistance, hypertriglyceridemia and increased risk of development of type 2 diabetes mellitus\textsuperscript{201}. Appropriate lipid homeostasis is thus vital for maintenance of health.

Hormones involved in promoting lipid accumulation include insulin and cortisol, which act by inducing expression of enzymes and proteins involved in lipid storage\textsuperscript{202}. On the other hand, growth hormone, testosterone and catecholamines induce lipolysis and cause lipid mobilization\textsuperscript{202,203}. Estrogen may play a role in location of lipid deposition but results are inconclusive regard possible cellular mechanisms\textsuperscript{188,204}. Progestin and estrogen were found to induce preadipocyte differentiation \textit{in vitro} and another study revealed estrogen maintains this function \textit{in vivo}\textsuperscript{205-207}. Adiponectin may play a role in maintaining adipocytes ability to store lipids, exhibited by adiponectin overexpressing leptin deficient mice with increased subcutaneous fat but smaller adipocyte size\textsuperscript{208}.

1.4.3 Lipid metabolism is regulated by endocrine signals

A vital function of the WAT is serving as a safe storage site for lipids in the form of triglycerides, which can then be converted to fatty acids and used as energy for exercise, during fasting or starvation. The rates of storage and lipolysis are tightly regulated by endocrine signals.
1.4.3.1 Regulation of lipolysis by insulin and catecholamines

Lipolysis is the hydrolysis of triglycerides stored in intracellular lipid droplets. Access to the triglycerides within the droplet is regulated by perilipin, a lipoprotein that coats lipid droplets and is involved in regulating lipolysis\textsuperscript{209}. In adipose tissue, the products of lipolysis are non-esterified fatty acids, which can then be exported from the adipocytes for translocation to another tissue where they can undergo β-oxidation for production of ATP or be used to produce other biologically necessary lipids. In adipocytes triglycerides are hydrolyzed primarily by two enzymes: adipose triglyceride lipase converting triglycerides to diacylglycerols, and hormone sensitive lipase,\textsubscript{,} hydrolyzing diacylglycerols to monoacylglycerols\textsuperscript{210,211}. Adipose triglyceride lipase mRNA expression is increased by glucocorticoid action and during fasting, and is decreased by insulin and food intake\textsuperscript{209,212,213}. However, as these lipases activities are highly regulated by post-translational modifications gene expression changes do not imply changes in lipase activity\textsuperscript{209}. The physiological role of lipolysis is to provide fatty acids as an energy source during prolonged fasting when insulin and catecholamine levels are low or under acute stress initiated by catecholamines\textsuperscript{214}. Intracellular cyclic AMP (cAMP) stimulates protein kinase A, which phosphorylates both hormone sensitive lipase and perilipin thereby activating lipolysis\textsuperscript{215}. β-Adrenergic stimulation by catecholamines causes production of cAMP via stimulatory G-proteins, whereas insulin signalling activates phosphodiesterase 3B which decreases cAMP levels\textsuperscript{214}. Thus the rate of lipolysis is balanced by endocrine signals.
1.4.3.2 Regulation of de novo lipogenesis by insulin

As lipids are a necessary component of all cellular life there are mechanisms by which organisms have evolved to produce lipids from other carbon sources like carbohydrates or protein metabolites. In humans under normal conditions, the majority of stored triglycerides are obtained from fatty acids in the diet, but a small amount (1-5%) is produced by the liver or (1-2%) in the adipose tissue\textsuperscript{214,216}. After excess carbohydrate ingestion, the liver can synthesize esterified fatty acids and triglycerides that are then secreted as very low density lipoprotein (VLDL) particles\textsuperscript{217}. Once in the circulation, fatty acids can be released from VLDLs by hydrolysis of lipoprotein triglycerides by lipoprotein lipase found on the surface of epithelial cells in capillaries\textsuperscript{217}. Cycling of fatty acids from adipose tissue to liver is accomplished by production and secretion of non-esterified fatty acids (bound to circulating proteins) and from the liver to adipose tissue via VLDLs\textsuperscript{217,218}. The synthesis of fatty acids is also hormonally regulated. Insulin acts to increase fatty acid synthesis in the liver during carbohydrate excess by increasing cAMP and increasing sterol regulatory binding element-1c (SREBP-1c) expression, which is a transcription factor known to affect expression of genes involved in fatty acid and triglyceride synthesis\textsuperscript{217,219}. This effect is decreased during fasting when glucagon levels increase, which decreases cAMP levels in hepatocytes\textsuperscript{219}. Understanding the relationship between lipid metabolism and the endocrine system are vital for discoveries of novel therapeutic targets for diseases with defects in either system. Dietary interventions that affect hormones regulating lipid metabolism,
like insulin, may provide further insight into how these biological systems interact.

1.5 Caloric restriction slows ageing and improves metabolic outcomes

1.5.1 Caloric restriction and lifespan extension

In 1935, diet restriction in rats was identified as the first experimental method to extend lifespan\textsuperscript{220}. Since this initial discovery of lifespan regulation by food intake, it has been replicated and demonstrated that from yeast to mammals restricting food or caloric intake has a positive effect on longevity\textsuperscript{221}. Caloric restriction, defined generally as a reduction in caloric intake from 20-60\% less than theoretical energy requirements (while avoiding malnutrition), has become an exciting diet intervention to study due to its consistent longevity inducing phenotype. However, there are cases where caloric restriction in certain strains of mutant yeast or mice of different genetic backgrounds have not led to increased lifespan and suggest the effect is genotype dependent\textsuperscript{222-224}. Currently studies in non-human primates, one at Wisconsin National Primate Research Centre (WNPRC) in Rhesus macaques (\textit{Macaca mulatta}) and the other at the National Institute for Aging (NIA), are inconclusive whether caloric restriction has a positive effect on lifespan in primates\textsuperscript{225-228}. Of the three primate studies reported, only one has found a statistically significant difference in all-cause mortality on its own. However, after a recent meta-analysis including data from all three studies only a decrease in age-related and not all-cause mortality is statistically significant\textsuperscript{225-228}. It should be noted that the studies differed in terms
of the origin of the animals included, age of diet initiation and the caloric restriction protocols themselves, which may contribute to the variation in results\textsuperscript{226,227,229}. Consequently whether or not caloric restriction is likely to increase human lifespan or not remains under debate\textsuperscript{228,230}. These studies are also grossly underpowered and in the NIA study the control diet is not truly characteristic of \textit{ad libitum} \textsuperscript{226-228}. The cost and logistics of caloric restriction studies in humans and non-human primates make it extremely difficult to determine mechanism and physiological effects of long-term caloric restriction. Thus the use of a mouse model is more economically feasible and can provide more detailed information about how caloric restriction exerts its beneficial effects.

1.5.2 Improved glucose tolerance in caloric restriction

Caloric restriction is extremely effective at improving glucose regulation from rodents to non-human primates and humans\textsuperscript{231-240}. The effects on glucose homeostasis include increased tolerance to a given glucose load compared to \textit{ad libitum} or free-fed controls as well as decreased fasting glucose and insulin and increased whole-body insulin sensitivity\textsuperscript{231-240}. These effects are seen in obese, diabetic and healthy individuals\textsuperscript{239,241-243}. Diets that exploit the caloric restriction paradigm provide a promising treatment option for improving glucose management without the need for drugs.
1.5.3 Caloric restriction and age-related disease risk

Expected lifespan for humans has increased dramatically over the last hundred years from 45 to at least 77 years in many developed countries\textsuperscript{244}. The effect on healthspan, lifespan without chronic disease, has not been as great exhibited by the growing burden of chronic diseases such as type 2 diabetes, obesity, cardiovascular disease, Alzheimer’s disease, respiratory diseases and cancers\textsuperscript{245}. Thus, regardless of whether or not a positive indication is found for lifespan extension the study performed at the WNPRC found significant decreases in age-related diseases (defined as cancer, diabetes, arthritis, diverticulosis and cardiovascular disease)\textsuperscript{227}; an economically important outcome that could greatly benefit quality of life for many people if this diet or a mimetic can be translated to human interventions. Some benefits to the caloric restriction diet have already been illustrated in healthy non-overweight humans. For example several surrogate risk factors associated with atherosclerosis and cardiovascular disease, a leading cause of age-related death, were significantly reduced after only 6 months of 25\% calorie restriction in both men and women in one study\textsuperscript{246,247}. Additionally, placing obese or diabetic patients on a calorie-restricted diet has many positive effects including greatly improved glycemic control and improved lipid profiles\textsuperscript{230,238,243,246,248,249}. A 30\% calorie restricted diet, compared to individual pretrial food intake, was found to improve memory in elderly adults (between age 50 and 80) after just 3 months, which the authors suggest may be due to decreased circulating insulin levels\textsuperscript{250}. With the many beneficial outcomes as a result of even short-term caloric restriction on disease
risk factors or even in healthy subjects it is not a surprise that there is so much
research poised to discover the molecular mechanisms by which this diet exerts
its physiological effects. The ideal scenario might be the discovery of caloric
restriction mimetics that confers a similar delay and/or prevention of age-related
diseases.

1.5.4 Longevity related signalling pathways induced by caloric restriction

Several important pathways involved in the extension of lifespan by
caloric restriction have been identified in *C. elegans*, *D. melanogaster* and mice,
but the exact mechanisms by which restriction of food intake increases lifespan
have yet to be elucidated. To date, caloric restriction studies implicate cellular
nutrient-sensing pathways, including insulin/IGF-1-like signalling, kinase target of
rapamycin (TOR), AMP kinase, and sirtuins as active regulators of the lifespan
extension seen in caloric restriction\textsuperscript{251-260}. Other important cellular mechanisms
have also been implicated in caloric or glucose restriction in lifespan extension of
*C. elegans*, including autophagy and mitochondrial respiration\textsuperscript{261-263}. To
corroborate, a large-scale study of gene expression changes as a result of
ageing in multiple mouse tissues shows a decrease in genes involved in energy
metabolism\textsuperscript{264}. Meta-analysis of 27 datasets of gene expression profiles
obtained from mouse, rat and human tissues found a similar signature as a
result of ageing\textsuperscript{265}. This indicates the use of mouse models is well suited for
aging research as discoveries are likely to be clinically significant.
Genetically engineered mice provide a prime tool in order to determine mammalian mechanisms by which caloric restriction induces a longevity phenotype. Mice lacking \textit{Irs1}, a gene encoding an intracellular insulin receptor substrate, have metabolomes with remarkable similarities to mice on caloric restriction\textsuperscript{266}. These data suggest a potential role for insulin as a mediator of caloric restriction induced longevity. However, mice with mutations in the somatotropic axis (growth hormone/lgf1/insulin), such as the growth hormone releasing hormone knockout mice, which have genetically increased lifespans have a further extension of lifespan when placed on caloric restriction. This additivity of the response is taken to imply that other molecular mechanisms may be involved\textsuperscript{267}. Similarly, there is evidence from \textit{C. elegans} and \textit{D. melanogaster} suggesting caloric restriction and insulin signalling mutants have additive effects on lifespan, suggesting the two manipulations function through independent molecular mechanisms\textsuperscript{268-272}. However, these conclusions are not comprehensive and draw from mutants of only one of the many downstream targets of insulin signalling, the ortholog of the forkhead box transcription factor, \textit{foxo3a}. Other studies, some in mammals, suggest they have common mechanisms\textsuperscript{240,251,268,273}. The specific effects of circulating insulin levels on lifespan and caloric restriction have never been evaluated. Key genetic investigations are needed to determine undeniably whether caloric restriction extends life in mammals by reducing circulating insulin.
1.5.5 Hormonal alterations under caloric restriction

Many physiological changes occur as a result of caloric restriction including alterations in circulating hormone levels to mediate tissue responses under energy restriction. Some of the most consistent findings include decreases in insulin, triiodothyronine (T3), leptin, Igf1, growth hormone, pro-inflammatory cytokines and increases in cortisol (corticosterone in rodents) and adiponectin\textsuperscript{239,274}. It is evident that the main effect for reduction in hormones like T3 and leptin would act to decrease energy expenditure in an energy deficient state by lowering resting body temperature\textsuperscript{275-277}. Anabolic hormones like insulin, Igf1 and growth hormone act to increase cell proliferation and are associated with tumorigenesis; mice with defects in the somatotropic axis have decreased cancer incidence and longer lifespans\textsuperscript{267,278-285}. Decreased inflammatory molecules and increases in cortisol levels result in lower systemic inflammation and age-related dysregulation of inflammatory cytokines\textsuperscript{286-291}. Increases of adiponectin are likely involved in increasing insulin sensitivity under caloric restriction\textsuperscript{292,293}. Determining which of these effects are required for the beneficial effects of caloric restriction is necessary for the potential development of calorie restriction mimetics.

1.5.6 Effect of caloric restriction on body composition

Calorie restricted diets have profound effects on body composition, as might be expected. Mice and humans on this diet exhibit decreases in lean and fat mass as well as decreased body fat relative to body weight\textsuperscript{233,248,294-296}. The
decrease in body fat is seen ubiquitously in caloric restriction literature. The exception is for a very mild (5%) and very short (4-week) caloric restriction diet given to female mice where fat mass was increased, lean mass decreased and overall body mass maintained\textsuperscript{297}. Interestingly, it has been reported that the ratio of lean to fat mass lost depends on age of caloric restriction initiation, where fat mass is lost more in young mice and lean mass in aged mice\textsuperscript{233}. The amount of lean or fat mass maintained during a 40% restricted diet in multiple inbred mouse strains were correlated with lifespan extension\textsuperscript{298}. Liao et al. found that a reduction in fat stores was negatively correlated with lifespan extension in the CR mice, illustrating that strains with the smallest reduction in fat mass had significantly increased likelihood of having longer lifespans\textsuperscript{298}. These findings suggest retaining a certain amount of fat storage is important for lifespan extension under caloric restriction and provides some insight into the genetic variation involved in the longevity phenotype\textsuperscript{298}.

1.6 Aim of thesis investigation

Caloric restriction, involving a 20-60% reduction in food intake, has numerous beneficial biological effects including significant improvements to glucose metabolism, decreased incidence of age-related diseases like cancer, diabetes and various neurological diseases as well as increases in lifespan\textsuperscript{230,247,274,299}. Insulin and insulin/Igf1-like signalling play significant roles in moderating invertebrate and mammalian energy, glucose and lipid homeostasis as well as food intake and lifespan\textsuperscript{251}. The beneficial effects of caloric restriction
on lifespan and healthy aging are primarily attributed to alterations in glucose and energy regulation, neuroendocrine and hormone level changes as well as decreases in body fat. Understanding the relationship between nutrient sensing hormones like insulin and the effects of restricted diet on aging and energy metabolism is key to identifying the interplay of diet and the endocrine system. Thus the thesis research performed was aimed at discovering the relationship between decreased circulating insulin levels and caloric restriction.

As long-term caloric restriction is accompanied by decreases in fasting insulin, it has yet to be explicitly determined whether a decrease in circulating insulin is required for improved glucose tolerance, insulin sensitivity and concurrent hormonal alterations. Hyperinsulinemia is prevented despite high-fat feeding in male \( \text{Ins1}^{+/+}:\text{Ins2}^{-/-} \) mice and hyperinsulinemia is not seen in female \( \text{Ins2}^{-/-} \) mice regardless of \( \text{Ins1} \) alleles\(^{85}\). The ability of female \( \text{Ins1}^{+/+}:\text{Ins2}^{-/-} \) and \( \text{Ins1}^{+/-}:\text{Ins2}^{-/-} \) mice to maintain low, unchanging insulin levels despite high-fat feeding suggests the insulin secretion profile of these mice do not respond conventionally to diet changes. Additionally the \( \text{Ins1}^{+/-}:\text{Ins2}^{-/-} \) mouse has the fewest \( \text{Ins} \) alleles that permit sufficient, albeit very low, circulating insulin without the development of diabetes or hyperglycemia.

Caloric restriction is also hypothesized to promote healthy glucose homeostasis and lifespan extension by decreases in fat mass\(^{300}\). Insulin is known to increase body mass and fat storage. It is not known what role circulating insulin plays in decreased fat storage during long-term caloric restriction. No study has been able to separate caloric restriction and circulating
insulin effects on fat and body composition. Male $\text{Ins}^+/+:\text{Ins}^2/-$ mice exhibited hypertrophy of epididymal fat on high-fat diet and increased fat to lean mass ratio, which were abrogated in the $\text{Ins}^+/+:\text{Ins}^2/-$ mice$^{85}$. This difference was attributed to increased WAT $Ucp1$ expression, increased energy expenditure and prevention of hyperinsulinemia$^{85}$. These data suggest decreased insulin production in this model lead to changes in body composition, energy expenditure and gene expression.

As female $\text{Ins}^2/-$ mice have very low insulin but do not become hyperinsulinemic on a high-fat diet, they may serve as a model to distinguish the insulin-mediated effects of caloric restriction. Specifically this thesis aimed to determine the effects of caloric restriction and $\text{insulin}$ gene dosage on glucose homeostasis, metabolic hormone levels, energy expenditure and body composition. Previous studies on female $\text{Ins}^2/-$ mice in our lab were underpowered and used a different diet$^{85}$. Therefore it is still prudent to perform all experiments on both $\text{Ins}^+/+:\text{Ins}^2/-$ and $\text{Ins}^+/+:\text{Ins}^2/-$ as the different diet used in this study may alter the insulin phenotype of these mice. The use of both genotypes increases the likelihood one of them will have unaltered circulating insulin on caloric restriction.
Chapter 2: Materials and methods

2.1 Experimental animals

Animal procedures were approved by the University of British Columbia Animal Care Committee and in accordance with the guidelines set out by the Canadian Council for Animal Care. The $Ins2^{-/-}$ mouse model used was created at INSERM by J. Jami and colleagues as is described by Duvillie et al.\textsuperscript{90}. Our study focused on female mice, since male $Ins1^{+/+}:Ins2^{-/-}$ mice occasionally develop spontaneous, persistent hyperglycemia between 8-13 weeks of age. All mice in the study had a mixed background that was predominantly C57BL/6. Mice were fed a commercial chow, Teklad Diet LM485 manufactured in Madison, WI (chow details provided in Table 2.1). At 13 weeks of age the female $Ins1^{+/+}:Ins2^{-/-}$ and $Ins1^{+/+}:Ins2^{-/-}$ littermate mice were randomly placed into either an ad libitum-fed (AL) or a caloric restriction (CR) group fed 60% of food intake of the same genotype AL controls and housed individually to ensure equal feeding for CR mice. A fifth group was introduced of $Ins1^{+/-}:Ins2^{-/-}$ mice being fed 60% of $Ins1^{+/-}:Ins2^{-/-}$ AL group to separate genotype from food intake effects in certain measurements. Mice weights and food intake were measured weekly until 80 weeks thereafter they were weighed every other week. To measure food intake of AL group, the amount of food removed from the cage hopper was weighed weekly with any spillage subtracted. The CR mice were given 60% of food consumed by the genotype matched AL group using an automated feeder (Fish Mate F14 Aquarium Fish Feeder) refilled twice a week that would drop weighed food pellets into cage hopper in three quasi-equal “meals” during the dark phase.
The meals were dispensed two, five and eight hours after initiation of dark phase of the 12-hour light/dark cycle (lights on at 7 AM). Feeders were checked every morning by experimenter or animal technician to verify all food pellets had dropped. If they hadn’t the mice would be given the remaining food. Mice were housed in specific pathogen free conditions on ventilated (50 air changes per hour), autowater Eherent mouse cage racks at ambient room temperature of 21°C.

2.2 Glucose homeostasis, insulin sensitivity and plasma analysis

Mice were fasted for 4 hours during light phase initiated at 8 or 9 AM to provide a postprandial state for glucose homeostasis measurements and blood sampling. All CR mice meal times were shifted forward 4 or 5 hours so that their final meal was given 1 hour prior to fasting initiation. At 6, 12 and 18 months of age, fasting and intraperitoneal glucose-stimulated (2g/kg) insulin secretion were measured, as were the blood glucose response to intraperitoneal administrations of glucose (2g/kg) or an insulin analog (0.75U/kg of Humalog; Eli Lilly, Indianapolis, IN, USA). Blood glucose measurements were assessed with OneTouch Ultra2 glucose meters (LifeScan Canada Ltd, Burnaby, BC, Canada), plasma insulin levels were determined by mouse ultrasensitive insulin ELISA kit (Alpco Diagnostics, Salem, NH, USA), according to manufacturer’s instructions. Area under the curve was calculated to determine statistical differences in the glucose and glucose-stimulated insulin secretion tests. The MAGPLEX mouse magnetic bead panel assay (Millipore, St. Charles, MO, USA) was used to measure leptin, ghrelin, resistin, interleukin 6, glucose-dependent insulinotropic
polypeptide, peptide YY, and glucagon levels in fasting plasma samples collected from 48 week-old mice, in accordance with manufacturers’ instructions.

2.3 Body composition and energy expenditure

In order to determine *in vivo* body composition, dual-energy X-ray absorptiometry (DEXA; Lunar PIXImus densitometer; GE Medical Systems LUNAR, Madison, WI, USA) was used in 48-week old mice. Organ and fat depot contributions to lean and fat mass were assessed by gross dissection and measurement of wet-mass of 66-week old and 106-week old mice. Energy expenditure was examined in 48-week old mice using metabolic cages (PhenoMaster; TSE Systems, Bad Homburg, Germany). Mice were housed and acclimated in experimental room for 3 days. Cages were then placed in the environment-controlled chambers at 21ºC for oxygen and carbon dioxide gas exchange measurement, physical activity by beam breaks, and food intake for the AL group over 72h. Data from the first light phase were excluded. The remaining data from two full light cycles and three dark cycles were averaged. Total energy expenditure was calculated as previously described301.

2.4 Statistical analysis

Two-way analysis of variance (ANOVA) was performed with genotype and diet as the factors for a given measurement at a specific age. If a significant interaction was found, one-way ANOVAs with multiple comparisons were used instead to compare AL *Ins1*+/−:*Ins2*−/−, CR *Ins1*+/−:*Ins2*−/−, AL *Ins1*+/*:Ins2*−/−, and CR
Ins1\textsuperscript{+/−}:Ins2\textsuperscript{−/−}, with Bonferroni corrections applied. Statistics were done using Prism (version 6.0a; GraphPad Software, La Jolla, CA). The effect of age was not determined due to some mice having missing data points. Data presented are mean ± standard error of the mean. P values <0.05 were considered statistically significant.

2.5 Tables

Table 2.1 Teklad LM-485 chow diet macronutrient composition

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein</td>
<td>19.1%</td>
</tr>
<tr>
<td>Fat (ether extract)</td>
<td>5.8%</td>
</tr>
<tr>
<td>Carbohydrate (available)</td>
<td>44.3%</td>
</tr>
<tr>
<td>Crude Fiber</td>
<td>4.6%</td>
</tr>
<tr>
<td>Neutral Detergent Fiber</td>
<td>13.7%</td>
</tr>
<tr>
<td>Ash</td>
<td>6.1%</td>
</tr>
<tr>
<td>Energy Density</td>
<td>3.1kcal/g</td>
</tr>
<tr>
<td>Calories from Protein</td>
<td>25%</td>
</tr>
<tr>
<td>Calories from Fat</td>
<td>17%</td>
</tr>
<tr>
<td>Calories from Carbohydrate</td>
<td>58%</td>
</tr>
</tbody>
</table>
Chapter 3: Glucose homeostasis, body composition and metabolic hormone profile of *ad libitum* and calorie-restricted *Ins2*-/ female mice

3.1 Caloric restriction decreases body mass

Energy restricted mice, rats and humans are known to lose body mass when placed on a restricted diet after a period of energy excess\(^{234,235,239,240,248,294,302-304}\). The body weight of female *Ins1*+/*Ins2*-/ mice was indistinguishable from that of *Ins1*+/:*Ins2*-/ before initiation of caloric restriction (Fig 3.1A). Mice fed *ad lib.* continued to increase their body weight until 30 weeks of age, after which their weight stabilized (Fig 3.1A). Unsurprisingly, mice on caloric restriction initially lost weight for 5 weeks when first placed on the diet at 13 weeks of age and stabilized between 15-20% less than their littermate *ad lib.* controls (Fig 3.1A). *Ins1*+/:*Ins2*-/ mice weighed slightly less than *Ins1*+/:*Ins2*-/ mice until about 50 weeks of age. (Fig 3.1A). A pair-fed study where calorie-restricted *Ins1*+/:*Ins2*-/ were fed 60% of the *ad lib.* *Ins1*+/:*Ins2*-/ and thus fed the same as calorie-restricted *Ins1*+/:*Ins2*-/ abolished the difference in body weights of calorie-restricted mice(Figs 3.1B). This suggests that the body weight difference observed in calorie-restricted *Ins1*+/:*Ins2*-/ and *Ins1*+/:*Ins2*-/ mice are a result of a very small increase in food intake of *ad lib.* *Ins1*+/:*Ins2*-/ causing a very small, not statistically significant increase in food intake of calorie-restricted *Ins1*+/:*Ins2*-/ mice (Fig 3.2).
3.2 Caloric restriction lowers fed and fasting blood glucose and increases glucose tolerance

The effects of a calorie-restricted diet on blood glucose have been well documented. In non-human primates, humans, rats and mice fasting blood glucose is decreased and glucose tolerance increased under caloric restriction compared to control-fed animals. Similarly, *Ins2−/−* mice on caloric restriction had decreased fasting and fed blood glucose levels with no difference between ages (Fig 3.3A,B). The restricted mice also had increased glucose tolerance when challenged with an intraperitoneal glucose challenge (2g/kg) (Fig 3.4A). However, no differences in blood glucose levels or glucose tolerance were observed between mice with only one or both *Ins1* alleles at all three ages tested (Fig 3.4A). It was previously noted that *Ins1+/−/Ins2−/−* mice have no difference in glucose tolerance compared to wildtype mice. These data taken in combination suggest that one *Ins1* allele is sufficient to compensate for the lack of other *Ins* genes in maintaining euglycemia in mice. In fact, in previous studies it was demonstrated that male or female *Ins2−/−* mice maintained euglycemia and glucose tolerance when challenged with a high fat diet. However, those results were obtained in a conventional mouse facility and may not apply to newer pathogen-free facilities due to potential microbiota effects on metabolism.
3.3 Effects of caloric restriction and \(\text{Ins1}\) gene dosage on glucose-stimulated insulin secretion

In most studies where mice, rats, non-human primates or humans are placed on a CR diet there is either a decrease or no change in glucose-stimulated insulin secretion for a given glucose load\(^{232,236,295}\). In our study, no differences were seen in insulin secretion regardless of genotype or diet (Fig 3.5B). This is further evidence that a single \(\text{Ins1}\) gene is sufficient to produce enough protein to maintain glucose homeostasis and that mouse islets are able to maintain minimum levels of insulin production regardless of \(\text{Ins}\) gene dosage.

3.4 Caloric restriction increases rate of return to baseline glucose levels post insulin injection

One of the main beneficial effects of caloric restriction at least in terms of its effects in obese or type 2 diabetic patients, is its insulin sensitizing effect. In the large majority of studies caloric restriction is accompanied by a reduction in insulin resistance. In the \(\text{Ins2}^{-/-}\) mice, caloric restriction did not appear to increase insulin sensitivity, as measured by the glucose response to an intraperitoneal injection of insulin (Fig 3.4B). Instead, a response that could be interpreted as increasing insulin resistance was found (Fig 3.4B). For the first 30 minutes after 0.75U/kg insulin injection, all groups followed similar trajectories and reached similar minimal blood glucose levels around 3 mM (Fig 3.4B). After this minimal point all groups began increasing their blood glucose, likely countering the insulin effect (Fig 3.4B). However, the rate of return to baseline glucose was
much more rapid in the calorie-restricted mice as most of them reached fasting levels by 60 minutes compared to ad lib. mice that never returned to baseline glucose over the 120 minutes post injection measured (Fig 3.4B). More surprisingly, calorie-restricted mice often had higher glucose levels than their initial fasting levels by the 90-minute time-point (Fig 3.4B). This appears to be a strong counterregulatory effect in response to dropping blood glucose as opposed to insulin resistance as these mice maintain an initial response to insulin (Fig 3.4B). It is conceivable that the calorie-restricted mice encounter lower blood glucose concentrations than ad lib. controls on a daily basis due to long fasting times in between meals. Consequently they may be more primed to respond to drops in blood glucose levels more efficiently.

3.5 Age-dependent decrease in circulating insulin of Ins1<sup>+/−</sup>:Ins2<sup>−/−</sup> mice is abrogated by caloric restriction

Next, we assessed the effects of caloric restriction on circulating insulin levels in the context of partial insulin gene deletion. Unlike in most caloric restriction studies, this diet applied in our reduced insulin gene dosage mice did not decrease fasting circulating insulin levels and mice with only a single Ins1 allele were able to maintain similar insulin levels as those with two alleles (Fig 3.5A). As the mice aged, this compensation diminished as ad lib. fed Ins1<sup>+/−</sup>:Ins2<sup>−/−</sup> had lower circulating insulin at 53 than 27 weeks of age (Fig 3.5A). Additionally Ins1<sup>+/−</sup>:Ins2<sup>−/−</sup> had lower insulin compared to Ins1<sup>+/+</sup>:Ins2<sup>−/−</sup> mice at 53 weeks of age (Fig 3.5A). However, when placed on caloric restriction Ins1<sup>+/−</sup>:Ins2<sup>−/−</sup> mice
maintained similar insulin levels as 53-week old ad lib Ins1+/+:Ins2−/− mice and 27-week old Ins1+/−:Ins2−/− mice (Fig 3.5A). This suggests caloric restriction is preventing a drop in circulating insulin over age in the Ins1+/−:Ins2−/− mice. In most aging studies, there is a trend for increasing fasting insulin as well as insulin resistance that are also not seen in these reduced insulin gene dosage mice. It is possible that the lack of an effect of caloric restriction on lowering insulin is due to these mice having reached a physiological ‘insulin floor’ where any further reductions would be deleterious and result in a pathological diabetic-like state. Female mice with a similar C57Bl/6 or 129/Sv backgrounds have been reported to exhibit fasting insulin levels of 0.5-2ng/ml.

3.6 Caloric restriction in mice with reduced insulin gene dosage alters metabolic hormone levels

Next, we evaluated how caloric restriction may affect the levels of other metabolic hormones in mice with reduced insulin gene dosage. Similar to most studies, IGF1 levels were diminished during caloric restriction in both genotypes (Fig 3.6G). However, growth hormone levels no longer correlated with IGF1 levels in these mice as the calorie-restricted Ins1+/−:Ins2−/− mice had increased growth hormone compared to ad lib. controls and calorie-restricted Ins1+/−:Ins2−/− mice had similar levels to their ad lib. controls (Fig 3.6H). The satiety hormone peptide YY was not affected by diet nor genotype, but was affected by fasting in most groups (Fig 3.6E). The orexigenic hormone ghrelin was not found to be altered by genotype, diet or fasting in these mice (Fig 3.6D). No statistically significant
differences in interleukin-6 levels were seen, although fasting had a tendency to increase this hormone in all groups (Fig 3.6C). Another adipokine, resistin, was not found to have any differences between groups or after fasting (Fig 3.6F). Interestingly, leptin levels in both the fed and fasted states were significantly increased by caloric restriction with no difference due to genotype. This is the first study to find this diet to induce an increase in circulating leptin (Fig 3.6A). Similarly, caloric restriction lead to an increase in fed circulating GIP and fasted only in $Ins1^{+/−}:Ins2^{−/−}$ mice (Fig 3.6B). Fasting also significantly decreased GIP levels, at least in CR mice (Fig 3.6B). Caloric restriction related effects on endocrine signals appeared to be significantly altered in reduced insulin gene dosage mice.

3.7 Caloric restriction increases fat mass in mice with reduced insulin gene dosage

Caloric restriction is known to have a significant impact on body composition and leads to decreased body fat mass and often decreased lean mass. Paradoxically, in our study we found an increase in percent body fat and fat mass due to caloric restriction of $Ins1^{+/−}:Ins2^{−/−}$ mice (Fig 3.7B,C). This may also be interpreted as a prevention of decrease in fat mass normally seen under caloric restriction. Lean mass was significantly reduced in $Ins1^{+/−}:Ins2^{−/−}$ mice (Fig 3.7A). These in vivo data obtained by DEXA scan were corroborated by tissue collection. Significant increases in relative inguinal fat depot size in both $Ins1^{+/−}:Ins2^{−/−}$ and $Ins1^{+/−}:Ins2^{−/−}$ in 66 week-old calorie-restricted mice were
observed (Fig 3.9A). The relative BAT size was also nominally increased (p=0.0515) in 66-week old calorie-restricted \textit{Ins1}\textsuperscript{+/-}:\textit{Ins2}\textsuperscript{-/-} mice (Fig 3.9A). The effect on BAT size was more substantial in 106 week-old mice where both relative and actual inguinal and BAT size were significantly increased in calorie-restricted mice (Fig 3.9C,D). Relative gonadal fat depot size was also increased as a function of caloric restriction diet (Fig 3.9C). Other body composition changes included decreases in relative and actual liver and kidney sizes due to caloric restriction (Fig 3.9). Although unexpected and paradoxical, the increases in fat mass size are consistent with the increased leptin levels observed as it has been repeatedly shown that leptin levels are strongly correlated with WAT size. Subsequently determining whether lipid metabolism was being altered as a result of caloric restriction in these reduced insulin gene dosage mice became of interest. No difference in circulating fasting non-esterified fatty acids or cholesterol were observed (Fig 3.8A,B). However, there was a significant increase in plasma triglycerides (Fig 3.8C). These data are also surprising, given that almost every other caloric restriction study reports decreases in triglycerides and occasionally increases in circulating non-esterified fatty acids. Collectively, these data suggest that the normal effects of caloric restriction on fat mass and triglycerides are reversed when insulin levels are extremely low.
3.8 Caloric restriction decreases energy expenditure and alters circadian respiratory exchange ratio cycling

In an effort to determine how mice fed 40% less food could have increased body fat these mice were placed in metabolic caging to determine total energy expenditure and respiratory exchange ratio. The caloric restriction diet led to a decrease in daytime energy expenditure, but not nighttime energy expenditure, normalized to lean mass (Fig 3.10A, 3.11A). No difference in mean respiratory exchange ratio (RER) was observed, however there were differences in RER cycling (Fig 3.10C, 3.11B). These data imply that calorie-restricted mice utilize different energy fuels than the *ad lib* mice depending on the time of day.
3.9 Figures

A

Figure 3.1 A) Body mass over 80 weeks in *ad lib* and 40% calorie-restricted *Ins2−/−* female mice and B) body mass of mouse cohorts with pair fed-restricted animals over 1 year. Results are mean ± standard error of the mean with n= 20-25 per group for A and n=3-9 for B. Statistical significance indicated by ** for p<0.01, between mice of same genotype and § for p<0.05 between mice on caloric restriction.
**Figure 3.2** Food intake of female *ad lib.* or 40% calorie-restricted *Ins2−/−* mice over 80 weeks. Results are mean ± standard error of the mean with n= 20-25 per group.

**Figure 3.3** A) 4-hr fasted and B) fed blood glucose of female *ad lib.* or 40% calorie-restricted *Ins2−/−* mice. Results are mean ± standard error of the mean with n= 11-25 per group. Statistical significance indicated by ** for p<0.01; ***p<0.001, ****p<0.0001 between mice of same genotype.
Figure 3.4 A) Intraperitoneal glucose (2g/kg) tolerance test and B) insulin (0.75U/kg) tolerance test after a 4-hr fast in ad lib. or 40% calorie-restricted female Ins2−/− mice. Insets represent Area Under Curve (AUC). Results are mean ± SEM with n= 11-25 per group. Statistical significance indicated by * for p<0.05; **p<0.01; ***p<0.001 between mice of same genotype.
**Figure 3.5** A) 4-hr fasting plasma insulin and B) intraperitoneal glucose-stimulated (2g/kg) insulin secretion test following 4-hr fast in *ad lib.* and 40% calorie-restricted *Ins2^{-/-}* female mice. Insets represent Area Under Curve (AUC). Results are mean ± SEM with n= 11-25 per group. Statistical significance indicated by * for p<0.05 and by # for p<0.05 between *Ins1^{+/+}Ins2^{-/-}* *ad lib.* mice and all other groups.
Figure 3.6 Fed and 4-hr fasted A) leptin, B) glucose-dependent insulinotropic polypeptide (GIP) hormone, C) interleukin-6 (IL-6), D) ghrelin, E) peptide YY (PYY) and F) Resistin in 48 week-old, ad lib. or 40% calorie-restricted Ins2\(^{-/-}\) female mice. G) 4-hr fasted IGF-1 and D) growth hormone at indicated age. Results are mean ± SEM with n=4-25 per group. Statistical significance indicated by * for p<0.05; **p<0.01; ***p<0.001.

Figure 3.7 A) Lean mass, B) fat mass, C) body fat, E) bone mass density (BMD) of 48-week old Ins2\(^{-/-}\) female mice fed chow diet ad libitum or subjected to 40% calorie restriction starting at 13 weeks old. Each value represents mean ± SEM of data from 5-7 mice. Asterisks (*) denote level of statistical significance between mice of same genotype on different diets. * p<0.05, **p<0.01. § denotes statistical difference p<0.05 between groups on same diet with different genotypes.
Figure 3.8 4-hr fasting plasma A) non-esterified fatty acids (NEFAs), B) cholesterol and C) triglycerides in 48-week old Ins2-/- female mice fed chow diet ad libitum or subjected to 40% caloric restriction starting at 13 weeks old. Each value represents mean ± SEM of data from 3-6 mice. Asterisks (*) denote level of statistical significance between mice of same genotype on different diets. * p<0.05, **p<0.01.
**Figure 1**: Effect of diet on tissue mass and % body weight.

**A**. Percentage body weight of tissues.

**B**. Tissue mass (g).

**C**. Percentage body weight of tissues.

**D**. Tissue mass (g).

Ad libitum Ins1+/+:Ins2-/-

Ad libitum Ins1+/+:Ins2-/-

Calorie restricted Ins1+/+:Ins2-/-

Calorie restricted Ins1+/+:Ins2-/-

*p = 0.0515

** = p < 0.05

*** = p < 0.001
Figure 3.9 A) and C) Relative organ, B) and D) wet tissue masses of 66 and 106-week old (respectively) female $\text{Ins}^{1+/+}:\text{Ins}^{2-/-}$ and $\text{Ins}^{1+/+}:\text{Ins}^{2-/-}$ littermates fed chow diet *ad libitum* or subjected to 40% calorie restriction. Each value represents mean ± SEM of data, n=4-9. Asterisks (*) denote level of statistical significance (2-way ANOVA multiple comparisons) between mice of same genotype on different diets. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. § denotes statistical difference $p<0.05$ between groups on same diet with different genotypes. BAT, brown adipose tissue.

Figure 3.10 Mean light and dark phase A) energy expenditure normalized to lean body mass, B) energy expenditure and C) respiratory exchange ratio (RER) in 58-week old $\text{Ins}^{2-/-}$ female mice fed chow diet *ad libitum* or subjected to 40% caloric restriction starting at 13 weeks old. Results are mean ± standard error of the mean (SEM) obtained over two light and three dark cycles with n=3-4 per group. Statistical significance indicated by * for $p<0.05$; ** $p<0.01$; *** $p<0.001$. 
Figure 3.11 A) Energy expenditure normalized to lean body mass and B) respiratory exchange ratio (RER) in 58-week old Ins2−/− female mice fed chow diet ad libitum or subjected to 40% caloric restriction starting at 13 weeks old. Results are mean ± standard error of the mean (SEM) obtained over two light and three dark cycles with n=3-4 per group. Statistical significance indicated by * for p<0.05. Meal times indicated for calorie-restricted mice.
Chapter 4: Discussion

4.1 Caloric restriction has minimal effects on circulating insulin in reduced insulin gene dosage mice

The goal of the present study was evaluate the effects of caloric restriction in the context of reduced insulin gene dosage. The circulating insulin levels of $\text{Ins}1^{+/+}:\text{Ins}2^{-/-}$ and $\text{Ins}1^{+/+}:\text{Ins}2^{-/-}$ mice were very low, approximately 20-60% that of ad lib. fed wildtype mice of similar background. $\text{Ins}1^{+/+}:\text{Ins}2^{-/-}$ and $\text{Ins}1^{+/+}:\text{Ins}2^{-/-}$ mice maintain similar fasting insulin levels whether fed ad lib. or a calorie-restricted diet. However, at one-year caloric restriction prevented an age-related decrease in fasting insulin in $\text{Ins}1^{+/+}:\text{Ins}2^{-/-}$ mice. Comparatively, female $\text{Ins}2^{-/-}$ mice fed a high-fat diet also did not elicit changes in fasting insulin\(^85\). These data suggest the islets of these mice are not significantly affected by diet and are able to maintain physiological levels of insulin whether stressed with energy restriction or energy excess.

4.2 Effects of caloric restriction and reduced insulin gene dosage on glucose homeostasis

One of the most prevalent and consistent findings in animal models or human studies of caloric restriction is a significant improvement in glucoregulation and insulin sensitivity. In the present study, decreases in fasting glucose and increases in glucose tolerance were consistent with previous findings. In contrast these same mice had no glucose tolerance difference when challenged with a high fat diet\(^85\). Blood glucose levels in response to insulin
injection were significantly different between ad lib. and calorie-restricted animals. Under certain manipulations of the data, such as a calculation of area over the curve, it might appear as though caloric restriction decreased insulin sensitivity in these mice. Similar to the transient insulin resistance observed previously in young high fat fed Ins2−/− mice. However, this conclusion would be misplaced, as all mice reach a similar minimal blood glucose concentration. The major difference in the calorie-restricted animals' response was a rapid and substantial increase in blood glucose after this minimal glucose level. This minimal level begins to approach levels likely able to induce a hypoglycemic response. It is possible that due to long-term caloric restriction and regular exposure to long-term daily fasting where their blood glucose levels may approach glucopenic levels they have developed a more robust, effective counterregulatory response to decreasing blood glucose. It would be interesting to determine whether this effect is primarily due to catecholamines or glucagon.

Insulin secretion dynamics in response to glucose administration demonstrate β-cell glucose responsiveness. These data in conjunction with glucose tolerance measurement and an insulin sensitivity test provide a snapshot of whole-body glucose metabolism. Despite improved glucose tolerance under caloric restriction and no apparent differences in insulin sensitivity, no difference in glucose-stimulated insulin secretion was observed. This begs the question, how are these energy deficient mice more rapidly dispensing circulating glucose with no increases in insulin sensitivity with a similar insulin response to glucose? There are a few possible answers. The first
being that the maximal insulin response of calorie-restricted mice is missed in
the time intervals measured (as insulin has a short half-life ~4-6 minutes). The
amount of glucose injected due to body weight differences could be the reason
blood glucose concentration in the smaller, calorie-restricted mice is more rapidly
decreased. However, the difference in body weight is minimal (~20%) and the
difference in peak glucose concentration more significant (~40%). Another
possibility is that insulin independent glucose uptake is higher in the calorie-
restricted mice, such that the rate of glucose uptake is increased with no effect
on insulin sensitivity.

It has been previously reported that Gip exerts effects other than glucose-
dependent insulin secretion potentiation, including increasing murine skeletal
muscle glucose uptake independent of insulin\textsuperscript{309,310}. Similarly leptin is able to
increase 2-deoxyglucose uptake into several tissues, including skeletal muscle
and BAT, independent of insulin action\textsuperscript{108,170,311,312}. As increased fasting and fed
levels of leptin and Gip levels were observed as a result of caloric restriction in
low insulin mice it may be possible that these hormones are promoting insulin-
independent glucose uptake.

In most studies, the beneficial effects of caloric restriction on
glucoregulation are attributed to decreases in circulating insulin and leptin.
However, in our model, circulating insulin is affected only as a function of \textit{Ins1}
gene dosage, where 1-year old \textit{Ins1}^{+/-}:\textit{Ins2}^{-/-} mice have decreased fasting insulin
compared to \textit{Ins1}^{+/-}:\textit{Ins2}^{-/-} littermates. It is therefore possible that the \textit{Ins2}^{-/-} mice
are operating at a physiological ‘insulin-floor’ where any decreases could result
in a type 2 diabetes-like state where insufficient insulin is produced. This may be what occurs in ad lib.-fed male Ins1\textsuperscript{+/−}:Ins2\textsuperscript{−/−} mice that spontaneously develop persistent hyperglycemia (observation from breeding colony). Interestingly, caloric restriction instead prevented an age-related decrease in fasting insulin in Ins1\textsuperscript{+/−}:Ins2\textsuperscript{−/−} mice. These data suggest that other endocrine factors such as adipokines or enteroendocrine and sympathetic nervous system activity may have equally important functions in mediating beneficial glucoregulatory effects of caloric restriction in our model.

4.3 Paradoxical increase in both subcutaneous white and interscapular brown adipose tissue due to caloric restriction in Ins2\textsuperscript{−/−} female mice

It was well established that moderate to severe caloric restriction had very strong, rapid and consistent effects on body composition including decreases in body fat mass and content as well as decreases in lean mass\textsuperscript{233,248,294-296}. These effects were primarily attributed to decreases in energy intake, as it has been found that resting energy expenditure in calorie restricted animals is much higher than predicted by their decreased mass\textsuperscript{313}. However in this study, despite a relatively constant reduction in body mass maintained at ~15-20% less than ad lib. controls, mice on long-term caloric restriction sustained increases in total fat mass and content as well as decreases in lean mass. This could in part be attributed to the decreased energy expenditure per unit lean mass observed during the light phase in the calorie-restricted mice. Although paradoxical, especially in mice with genetically lowered anabolic insulin, where one might
have expected a more drastic fat loss, the increased circulating triglycerides suggest increased *de novo* lipogenesis from the liver and associated lipid trafficking. Integrating the metabolic cage data suggests that the circulating lipid profiles of these mice likely change depending on the time of day as RER cycles according to feeding time. As the plasma samples examined were obtained during the middle of the light phase, when the calorie-restricted mice are primarily using carbohydrates as fuel and the *ad lib.* mice fatty acids, it may be slightly less surprising that their circulating triglycerides are significantly higher at this time. Additionally it is very interesting to note that these mice have increased fasting leptin levels despite being on a calorie-restricted diet, which has been repeatedly shown to decrease circulating leptin levels\textsuperscript{234,274,295,314}. As fat mass and leptin increased in these mice on caloric restriction it is clear that calorie-restriction itself does not function directly to alter leptin levels. Instead caloric restriction likely acts indirectly by decreasing fat mass which would result in a decrease in leptin. This being the first study able to differentiate an energy restriction and fat mass effect on circulating leptin.

Mice on caloric restriction are known to have significant gene expression changes, especially genes encoding glucose, lipid and mitochondrial metabolism\textsuperscript{315}. In WAT of calorie-restricted rats there is an increase in proteins involved in fatty acid biosynthesis, glucose and lipid metabolism and mitochondrial biogenesis\textsuperscript{316,317}. Okita *et al.* also noted that their calorie-restricted rats had smaller white adipocytes and smaller WAT size\textsuperscript{316}. Thus it may be that these processes are also augmented in this model and the diet or lack of *Ins2*
permits for more efficient storage such that even on calorie-restriction these mice store more fat than their *ad lib.* littermates. We are only aware of a single other publication wherein energy restriction was reported to lead to an increase in body fat and simultaneous decrease in lean mass. It should be noted that the publication reported on female C57BL/6J mice (a similar background to the mice in our study) and that the ‘caloric restriction’ diet was short (3-4 weeks) and unrestrictive (5% caloric restriction).

A potential mechanism in fat accumulation in our female *Ins2*−/− mice on caloric restriction may include a strong Gip component. It is likely if glucose was administered orally instead of injected into the peritoneal space the insulin secretion profile would differ between that *ad lib.* and calorie-restricted mice due to the significant difference observed in their fed Gip levels. Insulin has been found to inhibit Gip secretion in humans and rats, suggesting that *Ins2*−/− mice might be more primed for increased Gip production due to their low insulin levels. Gip also plays a role in glucose uptake and incorporation into lipids, increasing lipoprotein lipase activity, stimulating fatty acid synthesis as well as lipolysis in adipose tissue. Gip may also play an important role in maintaining circulating non-esterified fatty acids at appropriate levels during fasting. Lipolysis induced by Gip is inhibited by insulin. Several studies in Gip receptor knockout mice found that Gip has no effect on body weight, but Gip deficient mice had reduced adiposity on high fat diet. Even when placed on a chow diet Gip receptor knockout mice exhibit decreased body, visceral and subcutaneous fat. Thus, it is possible that the increased Gip found in calorie-
restricted \textit{Ins2}^{−/−} mice is acting on adipocytes to induce triglyceride storage and increasing WAT size. The Gip fat storage signal in conjunction with postprandial surges of insulin could strongly induce lipid storage mechanisms promoting efficient energy storage in WAT of our calorie-restricted mice.

\textit{Ins2}^{−/−} mice on caloric restriction maintain lower energy expenditure per unit lean mass during the end of the dark phase and beginning of light phase despite increased levels of leptin, known for its energy expenditure increasing effects. Unlike in the male \textit{Ins1}^{+/−}:\textit{Ins2}^{−/−} high-fat fed mice that exhibited increased energy expenditure compared to \textit{Ins1}^{+/+}:\textit{Ins2}^{−/−} littermates\textsuperscript{85}, no difference in genotype dependent energy expenditure was observed in the female \textit{Ins2}^{−/−} mice. This discrepancy is likely due to significant diet or housing differences. A potential mechanism for the observed decrease in energy expenditure due to caloric restriction is the induction of torpor. Torpor is a daily hypometabolic state that can be incurred by low ambient temperatures or by energy restriction in heterotherms near the end of night where body temperature is lowered below 30°C temporarily to decrease resting metabolic rate and thus energy expenditure\textsuperscript{332}. Torpor may also explain the increased BAT size observed in the calorie-restricted \textit{Ins2}^{−/−} mice. The mechanisms by which torpor is controlled are not fully elucidated but a role for leptin and the arcuate nucleus have some merit\textsuperscript{333,334}. Leptin has been shown to blunt the entrance of torpor in calorie-restricted mice, whereas NPY injected into the brains of Siberian hamster initiates a torpor-like state\textsuperscript{277,335}. There are also leptin-independent mechanisms that induce torpor that likely involve glucose sensing in the brain\textsuperscript{304,336,337}. The
rapid return of body temperature to normal levels requires BAT stimulation, likely by activation of β-adrenergic receptor stimulation via the sympathetic nervous system\textsuperscript{334,338,339}. BAT is known to undergo hyperplasia as a result of sympathetic nerve stimulation usually in circumstances of low ambient temperatures\textsuperscript{172,340,341}. Although body temperature was not explicitly measured the energy expenditure profile exhibits the characteristics of daily torpor and when calorie-restricted mice were handled in the morning their body temperature was noticeably lower than \textit{ad lib.} mice by touch. The calorie-restricted mice were also quite lethargic at this time. It is possible that the daily induction of torpor, and the resulting exit of torpor requiring BAT stimulation, may be responsible for the significant increase in BAT mass seen in calorie-restricted mice as torpor-like energy expenditure cycle is not evident in \textit{ad lib.} mice. It would be interesting to verify body temperature cycling of these mice to determine if torpor is truly induced in these mice. Similarly whether leptin cycles according to daily energy expenditure in these mice to determine what likely mechanism is involved in torpor induction. Understanding BAT physiology is of clinical significance as it could provide methods for safely treating obesity by increasing energy expenditure.

4.4 Summary

The significant differences in response to caloric restriction observed in these mice compared to previous studies provides exciting opportunities to learn more about the interactions of diet and physiology. Significantly, caloric restriction prevented age-related decrease in insulin of \textit{Ins1}\textsuperscript{+/-}::\textit{Ins2}\textsuperscript{-/-} mice.
compared to Ins$^{1+/+}$:Ins$^{2-/-}$ littermates and the well established decrease in fasting insulin due to caloric restriction was not observed in this model. This suggests that the insulin reducing effect of caloric restriction is dependent on physiological need rather than a direct insulin reducing effect on the $\beta$-cell. The majority of effects observed were a result of caloric restriction and not $Ins$ gene dosage. These include decreased fasting and fed glucose, increased glucose tolerance without changing whole-body insulin sensitivity or insulin secretion dynamics, decreased daytime but not nighttime energy expenditure normalized to fat-free/lean body mass with no alterations in daily fuel use type. Although perhaps the most interesting results were those that were truly unexpected, such as the increased total body fat, with a primary increase in subcutaneous white and interscapular BAT in long-term calorie-restricted female $Ins^{2-/-}$ mice. This was accompanied by increases in circulating triglycerides, Gip and leptin levels. These novel data suggest caloric restriction itself does not directly alter leptin levels but does so by altering fat mass and that the glucoregulatory and fat storage outcomes typical of caloric restriction depend on decreasing circulating insulin levels.

4.5 Limitations and future directions

Grasping the relationship between the endocrine system and long-term diet on aging and energy metabolism is necessary to discover ways to prevent or slow the progression of age-related diseases. The endocrine system and specifically insulin are known to play significant roles in regulating aging.
Similarly caloric restriction is known to extend lifespan under most experimental conditions. Determining the physiological effect of caloric restriction on mice with reduced insulin gene dosage is poised to provide new insights on the interaction of this lifespan extending diet and endocrine regulation. The data acquired in this study provide the first step in understand how a long-term calorie-restricted diet alters the physiology of Ins2 deficient mice. A caveat of the study is that it was not possible to distinguish whether the paradigm altering results were primarily an effect of the mice lacking the Ins2 gene as there was no fully wildtype littermate control. This control could have provided some answers regarding the effects of caloric restriction on circulating insulin as the effects of this diet differ depending on mouse strain and genotype. Additionally it would be very interesting to determine whether the fat phenotype observed in these mice is indeed a function of the Ins2 knockout by the addition of wildtype controls. However, the logistics of including this control would have required extensive breeding colonies as it would require breeding double het Ins1\textsuperscript{+/−}:Ins2\textsuperscript{+/−} mice in order to maintain the gold standard of littermate controls. This would imply some genotypes would only arise with a 1:16 frequency not including a further split by sex. Additionally the number of mice per group would have been unmanageable for one person.

The majority of caloric restriction studies have been performed on male C57BL/6 mice. However in most longevity studies with genetically altered insulin-related proteins a more robust lifespan extension is observed in female mice. Additionally male Ins1\textsuperscript{+/−}:Ins2\textsuperscript{−/−} mice can on occasion spontaneously develop
hyperglycemia by 15 weeks of age. This phenotype developed after a switch from a conventional to a pathogen free animal facility after these mice were used previously by Mehran et al. 85 Using female mice has the caveat of estrogen cycling, which can also be altered in caloric restriction342. However estrogen cycling is not expected to have a significant impact on this study. Another minor caveat that simply implies the results may not be as readily extrapolated to males is due to the sexual dimorphic effects on body weight management, fat storage and lipid profiles181,343-345. Interestingly, in one study female mice on caloric restriction preferentially maintained subcutaneous fat over visceral fat whereas male mice lost both equally344.

Some of the methods for measurements of glucose metabolism were limited, including the insulin tolerance test as measure of insulin sensitivity as it does not distinguish tissue-dependent differences. However, in order to assess insulin sensitivity the most accurately requires a hyperinsulinemic-euglycemic clamp, which is usually a terminal procedure. As we endeavoured to follow our mice longitudinally, this option was not available.

There were also caveats with regards to measuring tissue expansion. For instance, wet tissue weight is an indirect indication of triglyceride content but does not distinguish functional changes in BAT.

There is no set method on how to best administer food under caloric restriction. In our study we aimed to best approach normal murine nocturnal feeding times. This is likely an improvement on other studies that normally feed their calorie-restricted animals one bolus food during light phase. However, no
effect on the lifespan extension effect of caloric restriction is seen due to altered feeding time, however energy expenditure and body temperature coupling are changed depending on feeding time, which may have had an impact on the results obtained³⁴⁶.

Many further exciting questions have arisen from the results of the work in this thesis. It might be prudent to determine the histology of several tissues, including determining the size distribution and mitochondrial content of white and brown adipocytes as this may denote altered function as well as distinguish hypertrophy from hyperplasia of adipocytes in the calorie-restricted mice. Immunohistochemical staining for Gip receptor in adipose tissue as well as determining K cell number may help establish role and source of increased circulating Gip in calorie-restricted mice. Insulin and Gip secretion could be measured in response to either re-fed or glucose gavaged animals to establish the physiological relevance of these adiposity signals to fat mass control in our model. Validation of torpor by circadian body temperature measurements and adrenergic receptor staining would provide a stronger basis for potential method of increased BAT size. Finally it would be interesting to determine expression levels of lipogenic and lipolytic in WAT, BAT and liver of these mice to further establish hypothesized mechanisms for paradoxical fat increases.

Despite the caveats and need for future studies the present work advances our understanding of how caloric restriction affects mice in a new endocrine context of reduced insulin gene dosage.
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