FACTORS CONTRIBUTING TO ALTERED INSULIN LEVELS OF PWD/PhJ AND WSB/EiJ WILD-DERIVED INBRED MICE

by

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Abstract

Insulin is a key hormone in the regulation of blood glucose. Type 2 diabetes (T2D) results from insufficient insulin-producing β cells in pancreatic islets or insufficient insulin secretion to maintain glucose homeostasis. Genetic variability is a major factor affecting type 2 diabetes (T2D) development. Two inbred mouse strains derived from wild caught mice, PWD/PhJ (PWD) and WSB/EiJ (WSB), have novel genetic variation relative to the classically-studied mice, C57BL/6J (B6), which will assist genetic studies. PWD and WSB mice exhibit high and low insulin levels, respectively, without insulin resistance and obesity. The goal of this thesis was to investigate potential mechanisms behind the altered insulin levels of the PWD and WSB mice.

We found evidence that factors affecting the insulin secretion pathway were altered in PWD mice. Specifically, these affect insulin secretion stimulated by nutrients, with possibly a minor factor affecting steps downstream of cell depolarization. These could contribute to the high insulin levels in PWD mice compared to B6 mice. There were no differences between PWD and B6 mice in islet structure or β cell mass.

For WSB mice, we found their pancreas and islets fail to grow after birth compared to B6 mice. This may contribute to their low insulin levels at later ages. However, WSB mice also exhibited low insulin levels at young age when their β cell mass was still similar to B6 mice. Their insulin secretion pathway was investigated. Surprisingly, while WSB mice exhibited low insulin levels in in vivo secretion studies, they secreted high insulin levels in in vitro studies. Thus WSB mice may also have physiological differences evident in vivo that reduce their insulin secretion. The physiological mechanisms for the altered insulin levels of these strains are regulated by their genetic factors. This suggests that discovery of these novel genetic factors
could provide new insights into processes that regulate insulin levels that may help lead to novel treatments T2D.
Preface

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I designed and conducted the experiments, performed data analysis, and wrote the manuscript and thesis. My supervisor Dr. Susanne Clee designed and supervised the project, and edited the manuscript and thesis. Our collaborator Dr. James Johnson provided helpful suggestions for our research, edited the manuscript, and his laboratory performed the perifusion experiments. Xiaoke Hu conducted perifusion experiments with my assistance. Subashini Karunakaran managed the mice breeding program.

All procedures were approved by the University of British Columbia Committee on Animal Care, and they were performed according to the Canadian Council on Animal Care guidelines (Animal Care certificates A08-0435, A08-0618, A12-0227, A12-0228).
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List of Abbreviations

T2D – Type 2 diabetes
SNP – Single nucleotide polymorphism
GWAS – Genome wide association studies
ATP – Adenosine triphosphate
SUR1 – Sulfonylurea receptor
PDX-1 – Pancreas duodenum homeobox 1
NF-kB – Nuclear factor kappaB
GLUT – Transmembrane glucose transporter
NADH – nicotinamide adenine dinucleotide
Acetyl-CoA – acetyl-coenzyme A
TCA – Tricarboxylic acid
PDH complex – pyruvate dehydrogenase complex
α-KG – α-ketoglutarate
FADH$_2$ – Flavin adenine dinucleotide
Kir 6.2 – Inward-rectifier potassium ion channel subunit
RER – Rough endoplasmic reticulum
F-actin – Actin filament
SNARE – Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
V-SNARE – Vesicle soluble N-ethylmaleimide-sensitive factor attachment protein
T-SNARE – plasma membrane target-soluble N-ethylmaleimide-sensitive factor attachment protein
SNAP25 – Synaptosomal-associated protein 25
VAMP2 – Vesicle-associated membrane protein
NBF1 – Nucleotide binding fold
GTP – guanosine triphosphate
GDP – guanosine diphosphate
CDC42 – Cell division control protein 42 homolog
PAK1 – Serine/threonine-protein kinase
CAV-1 – Caveolin-1
RAC1 – Ras-related C3 botulinum toxin substrate 1
RAB-3A – Ras-related protein 3A
RAB-27 – Ras-related protein 27
RALA – Ras-like GTPase family
ETC – Electron transport chain
KATP – ATP-sensitive potassium channel
ACS – Acyl-CoA synthase
LC-acyl-CoA – Long-chain acyl-CoA
CPT-1 – Carnitine palmitoyl transferase 1
CL – Citrate lyase
ACC1 – Acetyl-CoA carboxylate-1
MEM – NAD-dependent form of malic enzyme
DIC – Dicarboxylate carrier
MEc – NADP-dependent form of malic enzyme
KIC – Alpha-ketoisocaproic acid
BCAT – Branched-chain amino transferase
GDH – Glutamate dehydrogenase
B6 – B6
PWD – PWD
WSB – WSB
CAST – CAST/EiJ
HFD – High fat diet
ELISA – Enzyme-linked immunosorbent assay
DIA – Diazoxide
PA – Palmitate
AUC – Area under curve
IHC - Immunohistochemistry.
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Dedication

To my family
Chapter 1: Introduction

1.1 Prevalence of type 2 diabetes

Type 2 diabetes (T2D) is increasing at an alarming rate worldwide (5, 14, 23). Approximately 366 million people were diagnosed with diabetes in 2011 while, at its current rate, it is expected to increase to an estimated 522 million people by 2030 (5, 23, 76). Diabetes increases the risk of many health complications including kidney failure (72), heart problems, stroke, and blindness (23). This can shorten patients’ life expectancy by up to 10 years (23). T2D not only has a health impact on individuals and a social impact on their families, but it also has substantial economic consequences for the healthcare system (5). For example, it is predicted that diabetes will cost the Canadian health care system 16.9 billion dollars a year by 2020 (5, 14), whereas the costs of diabetes in United States alone are currently 612 million dollars per day (5).

1.2 Type 2 diabetes and its development

T2D is diagnosed when fasting blood glucose is elevated beyond the normal range. Insulin is the major hormone that results in clearance of blood glucose after a meal. Insulin is produced by β cells in the islets of Langerhans in the pancreas. T2D occurs if there is insufficient insulin to lower blood sugar levels. This can occur if there are too few β cells (62, 140) or if the β cells cannot secrete enough insulin (130). Thus a failure of β cell mass or function to meet the body’s needs is a key component of T2D development.

Many factors including age, lifestyle, and genetics can affect or promote T2D (70, 119). For example, age alone does not result in T2D but being older increases the risk. Some of the major lifestyle impacts on T2D development include reduced physical activity, increased
consumption of diets high in fat, and obesity (5, 23, 44, 62, 81, 140, 154). Obesity is a major risk factor in the development of T2D, and it is associated with insulin resistance in the body (5, 23, 62, 81, 104). Insulin resistance is when the body does not respond as well to insulin, requiring more insulin to achieve the same effect. One function of insulin is to suppress the breakdown of fat and promote its storage as triglycerides. However, under some circumstances, such as in an obesity, individuals can become resistant to insulin (33, 65, 96). As a result of insulin resistance, β cells are required to secrete more insulin to compensate, leading to hyperinsulinemia (23, 62, 104, 154). Meanwhile, other studies have shown that hyperinsulinemia may also be present before insulin resistance (28, 100). In this case, insulin resistance could act as a compensation for increased insulin (78, 120). It is still debatable whether hyperinsulinemia is present before insulin resistance or vice versa. Either way, as the β cells work harder to produce more insulin they can become exhausted (102). This exhaustion is associated with decreased insulin production and secretion, and also enhanced apoptosis of these cells resulting in the loss of β cell mass (5, 23, 62, 81, 104, 140, 154).

However, despite this knowledge, the processes causing T2D and why some people develop or do not develop T2D are not completely understood. For example, 80 percent of obese individuals do not develop T2D (5, 44, 50, 62). Genetic factors are also key determinants affecting T2D development, but most of these factors are unknown (5, 44, 50, 62). Diabetes can be caused by rare mutations of single autosomal dominant genes (54) such as the mutation of KCDJll1 and ABCC8 (73). However, for the majority of people, T2D is likely caused by the combined effects of single nucleotide polymorphisms (SNPs) in more than one gene in combination with environmental and lifestyle factors such as poor diet and insufficient exercise (5, 125). Genome wide association studies (GWAS) have identified the SNPs associated with
T2D through evaluating which SNPs are seen more often in diabetic patients relative to non-diabetic patients (5, 54). These SNPs each increase the risk of T2D by a small amount and thus are difficult to detect (5). The majority of these SNPs are predicted to affect β cells, either their function or mass (111). For example, SNPs within the following genes *KCNJ11, ADRA2A*, and *KCNQ1* are all predicted to influence the exocytosis of insulin from β cells (111). Therefore, the GWAS results are more evidence that β cells are critical to the development of diabetes.

### 1.3 Type 2 diabetes: diagnosis and treatment

Individuals may find no symptoms present until T2D is fully developed. There are, however, ways to identify whether individuals are normal, prediabetic, or diabetic. Prediabetic indicates that individuals’ blood glucose levels are above normal yet below the diabetic threshold when fasting. Pre-diabetic individuals are at high risk for T2D development (125).

Two tests are commonly used to determine whether individuals are diabetic (125). Both methods are very similar, as both require the individuals to be fasted for at least 8 hours prior to diagnosis, and they involve examination of blood glucose concentration from collected blood samples. The first method measures the fasting plasma glucose. Individuals with glucose levels of more than 6.1 mmol/L and less than 7.0 mmol/L (125) are considered to have impaired fasting glucose, or to be pre-diabetic. Individuals are considered diabetic when their fasting plasma glucose concentration is more than 7.0 mmol/L. The second, or oral glucose tolerance test, method is much slower but more accurate. This method measures blood glucose after fasting for at least 8 hours and again 2 hours after drinking a solution containing 75 grams of glucose. The individuals with impaired glucose tolerance, or prediabetic individuals, have blood glucose concentrations of more than 7.8 mmol/L and less than 11.1 mmol/L (125). If they are diabetic,
then they have glucose concentration of more than 11.1 mmol/L 2 hours after drinking the glucose solution.

Once individuals are diagnosed with diabetes, a treatment plan is put into action. Before the physicians prescribe any medication, patients are advised to have a lifestyle change (24). As mentioned above, lifestyle has a major impact on T2D development. Increasing physical exercise, having a well balanced diet, and weight control cannot only aid in patients with diabetes, but also help to prevent T2D development (56, 66). When a change in lifestyle fails to work, medications are prescribed (21, 24).

One type of medication increases the body’s sensitivity to insulin. Medications that combat insulin resistance include thiazolidinediones and metformin (21, 24). Thiazolidinediones prevent the release and circulation of free fatty acids into the blood stream hence lowering insulin resistance (21, 24). However, majority of this class of medication stimulates weight gain and increases the risk of congestive heart failure (87). Meanwhile, metformin primarily acts on the liver where it lowers the glucose production by the liver (137). This medication is effective in suppressing hyperglycemia resulting in reduced hyperinsulinemia and insulin resistance (137).

However, increasing the body’s insulin sensitivity may be insufficient to treat T2D if the β cells are not producing a sufficient amount of insulin. Sulfonylurea derivatives stimulate insulin secretion whereas insulin therapy directly increases insulin (149). Sulfonylurea derivatives include tolbutamide, gliclazide, glipizide, glimepiride and glyburide (125). Sulfonylurea drugs inactivate the adenosine triphosphate (ATP)-sensitive potassium channel in the β cell by binding to the sulfonylurea receptor (SUR1) (90). These drugs keep the potassium channel closed preventing the efflux of potassium ions, resulting in continuous depolarization of the β cell (103). The insulin secretion pathway will be described below, including how
depolarization stimulates insulin secretion of \( \beta \) cells. This results in chronically elevated insulin, which could contribute to insulin resistance, obesity and later \( \beta \) cell exhaustion (102).

Insulin therapy involves subcutaneous injection of insulin (145). This injected insulin is then transported to the blood to maintain glucose homeostasis. Insulin injection can be done via an insulin pen or an insulin pump (145). There are different types of insulin pen which includes rapid-acting insulin (1) (Apidra, Humalog, and NovoLog), regular or short-acting insulin (Humulin R and Novolin R), intermediate-acting insulin (Humulin N and Novolin N), and long-acting insulin (Levemir and Lantus) (34). Meanwhile insulin pumps only have the rapid-acting insulin type. These insulin types differ in the time it takes insulin to reach the blood, the time the insulin is in its most effective period, and the time the insulin could last (1). Insulin therapy is normally given to type 1 diabetics unless T2D patients are suffering from severe pancreatic damage and other drugs have failed to maintain glucose homeostasis (1). This therapy can also be used in combination with other anti-diabetic drugs (48). Insulin therapy is difficult because of the need for daily injections (48). This could be painful for patients and site infections have been seen (145). Accurately estimating insulin needs is also difficult for many patients (131). Too much insulin could lead to life-threatening hypoglycemia while too little insulin could lead to hyperglycemia, which can lead to the complications of diabetes (131).

Both of these insulin stimulating drugs can have harmful side effects. In the short term, diabetic patients may experience hypoglycemia from hyperinsulinemia, which can lead to death. This happens because both sulfonylurea drugs and inaccurate insulin injection can cause increased circulating insulin as they are not stimulated according to the amount of nutrients in the body. Insulin secretion is deregulated causing lowering of blood glucose below the tolerable level (149). In the long term, chronic hyperinsulinemia from these treatments may increase
obesity and insulin resistance (149). As mentioned above, hyperinsulinemia can induce insulin resistance as a compensatory effect. This is why newer medications are needed.

New classes of drugs have recently been developed that address some of these problems (1, 8). This includes SGLT2 inhibitors and GLP-1 analogues/DPP4 inhibitors (1, 8). SGLT2 prevents the kidney from reabsorbing glucose back into the blood (1, 8). Thus, glucose gets excreted with urine. GLP-1 analogues mimic the action of this incretin but can have a longer lasting effect (1, 8). This is because they have been modified so that the DPP4 enzyme site is blocked and thus prevents GLP-1 from getting degraded within 1 to 2 minutes (1, 8). Another class of drugs that act on the same pathway are DPP4 inhibitors to prevent the degradation of endogenous incretins (1, 8). However, no therapies have proven to be effective long term, thus new therapies that avoid some of the problems of the early drugs are still needed.

1.4 Pancreas structure and function

The key organ playing a role in T2D is the pancreas (122). The pancreas is made of an exocrine portion and an endocrine portion (122). The exocrine compartment consists of acinar cells and duct cells. Exocrine cells secrete enzymes into the digestive system (122). The endocrine component consists of islets of Langerhans (122). The pancreas is comprised of only 1 to 2 percent of pancreatic islets, but the islets are surrounded by a rich amount of blood vessels receiving 10 percent of the blood supply of the entire pancreas (111). Pancreatic islets contain α cells, β cells, delta cells, pancreatic polypeptide, and epsilon cells, each secreting a different hormone that performs a different function (122).

Pancreatic α cells secrete glucagon when blood glucose level falls (71). The key function of glucagon is to increase glucose back to a normal level during fasting. Glucose is an important
fuel, especially for the brain, where it is required (71). Therefore, it is important for glucagon to promote the conversion of glycogen stored in the liver and the skeletal muscles into glucose and to increase glucose production by the liver (71). Working together with pancreatic α cells are the pancreatic β cells (136). β cells account for the majority (60-80%) of the cells in a healthy islet (112). They are responsible for secreting insulin. Insulin is a hormone that lowers blood glucose. Delta cells form 10% of the pancreatic islets (122). These cells produce somatostatin which is a hormone that suppresses insulin and glucagon (12). Pancreatic polypeptide producing cells are less than 1% in the pancreatic islets (93). This hormone plays a role in regulating appetite, food intake, and pancreatic secretions after a meal (75). These four major endocrine cell types work together to maintain glucose level present in the blood. Epison cells are less than 1% of the pancreatic islet cells. They secrete ghrelin which is a hormone stimulating food intake (3, 148).

As the goal of this thesis is to understand changes in insulin levels, it will focus on β-cells and the insulin they produce.

Insulin molecules secreted by the pancreas travel via the bloodstream to the corresponding tissues such as the liver, skeletal muscle, and adipose tissue. In these tissues, insulin stimulates the translocation of glucose transporters to the plasma membrane, allowing glucose uptake into the tissues (49). This lowers blood glucose by increasing the uptake of glucose into the liver and skeletal muscles to store as glycogen or into adipose tissues as to store as triglycerides (23, 154).

Lowering blood glucose is important as hyperglycemia can lead to a process called glucotoxicity (125). Glucotoxicity occurs when a high level of glucose is accumulated in the bloodstream damaging tissues in the body (125). Chronic high glucose is what causes the complications of diabetes (125). For example, high glucose could damage the lining of blood
vessels leading to retinopathy and renal failure (113). High levels of glucose can also create a large amount of stress in the β cells (62). Increased glucose metabolism in β cells can produce reactive oxygen species, and these species damage cellular components such as pancreas duodenum hoemobox 1 (PDX-1) (125) and can activate nuclear factor kappaB (NF-kB) (82). PDX-1 regulates the production of insulin, maturation of β cells, and other critical processes (125). Meanwhile, NF-kB is a transcription factor that stimulates the apoptosis of β cells (82). Both of these components result in the loss of β cell mass, deteriorating a key component in blood glucose homeostasis (149).

1.4.1 Insulin production by the β cells

Insulin is synthesized as an immature precursor known as preproinsulin (55). The preproinsulin peptide is produced on the surface of rough endoplasmic reticulum (RER) (55). This primary molecule enters the RER and the amino terminal signal sequence is cleaved resulting in proinsulin molecule. Proinsulin is trafficked through the RER to the golgi apparatus where it gets sorted and packaged. The packaging step combines zinc, calcium, and proinsulin into a clatherin coated secretory granule (55). At this point the granule is at an immature stage. To become a mature secretory granule, an ATP-dependent proton pump on the granule pumps protons into the granule, acidifying the granule lumen (55). This mature granule is no longer coated with clatherin and consists of a dense core insulin crystal formed by a zinc and calcium-dependent condensation process (55). During maturation, proinsulin gets cleaved into equal ratio of insulin and C-peptide by proprotein convertase enzymes 1/3 followed by proprotein convertase enzyme 2 (55, 141). Mature or not, the granule enters the insulin secretion process when stimulated (55).
1.5 The insulin secretion pathway

Glucose is the major factor triggering insulin secretion from β cells (6, 31, 68). Glucose rapidly enters the blood stream as soon as food is consumed (68) and digested. Glucose reaching the pancreatic islets is taken up via the transmembrane glucose transporter, GLUT1 and GLUT3 in humans (90) or GLUT2 in mice (17). These glucose transporters allow glucose to enter the β cells and stimulate a chain of reactions to promote insulin secretion.

1.5.1 Metabolism of glucose by glycolysis

When glucose enters the cell, it is phosphorylated by glucokinase and is metabolized by glycolysis (111). Glycolysis is composed of 10 enzymatic steps resulting in 2 molecules of pyruvate, a net of 2 ATP molecules, and 2 nicotinamide adenine dinucleotide (NADH) (29, 86) (Fig 1.1). Glucose is only partially metabolized at this point.
1.5.2 Metabolism of glucose-derived pyruvate by the tricarboxylic acid cycle

The end products of glycolysis, pyruvate molecules, are transported into the mitochondria via a pyruvate carrier and are then converted into acetyl-coenzyme A (acetyl-CoA) (146). Acetyl-CoA is necessary for the entrance to the next key process in metabolizing glucose, the tricarboxylic acid (TCA) cycle (Fig 1.2) (86).

In continuation with the glucose metabolism, acetyl-CoA enters the TCA cycle (77, 86). This cycle begins with acetyl-CoA combining with oxaloacetate to form citrate (86). It proceeds
through a series of interconversions, resulting in ATP, reducing equivalents and oxaloacetate, which can re-enter the cycle. As shown (Fig 1.2), the citrate is converted to cis-aconitate and then to isocitrate (86). By removing a carbonyl group from isocitrate, α-ketoglutarate (α-KG) and an NADH molecule are formed (86). α-KG is then converted to succinyl-CoA, producing another NADH molecule. Succinyl-CoA is then converted into succinate (86). This reaction leads to the formation of either a GTP or an ATP molecule (86). The formation of fumarate from succinate produces a flavin adenine dinucleotide (FADH$_2$) molecule (86). Finally, fumarate is hydrolyzed to create malate which is then converted to oxaloacetate (86). This reaction produces an NADH molecule. Oxaloacetate returns us back to the beginning of the TCA cycle (86). All the NADH and FADH$_2$ molecules produced carry electrons into the electron transport chain leading us into the next key process of the insulin secretion pathway, ATP production by oxidative phosphorylation (128).
The TCA cycle contains a series of reactions in order to produce electron donors including NADH and FADH$_2$ molecules from glucose and other nutrients.

1.5.3 ATP production by the electron transport chain

The electron transport chain, embedded in the inner membrane of mitochondrion, is used to drive ATP production (86) (Fig 1.3). This transport chain is made of several large complexes comprised of ubiquinone, cytochrome c, and ATP synthase (86). The process begins with complex I where the NADH molecules produced in the TCA cycle are converted into NAD$^+$ to donate the electrons to ubiquinone (86). The electrons are passed from one complex to the next,
putting protons into the intermembrane space. These combined redox reactions are used to create a proton gradient across the inner membrane of the mitochondrial lumen with more proton ions localized in the intermembrane space. This proton concentration gradient is used by ATP synthase for the de novo production of ATP from ADP and inorganic phosphate (86).

1.5.4 Increased ATP leads to depolarization and the rise of calcium

The ATP molecules produced by oxidative phosphorylation exit the mitochondria into the cytosol of the cell (Fig 1.3). This increases the ratio of ATP to ADP molecules leads to closing of ATP-sensitive potassium channel (K\text{ATP}) (103). This potassium channel is made of four inward-rectifier potassium ion channel subunits (Kir 6.2) from \textit{Kcnj11} at the core surrounded with four SUR1 subunits from \textit{Abcc8} (114). Without any stimulation, this potassium channel is normally open allowing the efflux of potassium ions. An increased ratio of ATP to ADP molecules closes the K\text{ATP} channel and inhibits the efflux of potassium ions resulting in the depolarization of the β cell (112). This depolarization stimulates a voltage-sensitive calcium channel located in the plasma membrane to open allowing the influx of calcium ions (114). This stimulates the release of insulin into the bloodstream through the steps described next.

1.5.5 Release of insulin in response to depolarization

The release of insulin from granules involves five sequential processes: translocation, docking, priming, fusion, and exocytosis (7) (Fig 1.3). Translocation involves the transfer insulin-containing mature granules from the interior of the β cell to the actin filament (F-actin) near the plasma membrane along microtubules (143). The docking step requires disruption of the F-actin barrier and prepares the granules for the binding of vesicle SNARE proteins (v-SNAREs)
to the plasma membrane target-SNARE proteins (t-SNAREs) (74) to form the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes at the plasma membrane (74). The major v-SNARE on insulin granules is synaptobrevin. The t-SNAREs include synaptosomal-associated protein 25 (SNAP25) and syntaxin-1A (74). Three additional proteins, granuphilin, MUNC13(114), and MUNC18 (55) interact with syntaxin-1A in order to bind syntaxin-1A to SNAP25 and vesicle-associated membrane protein (VAMP2) (74). The v-SNAREs also form a complex with the calcium channel forming an excitosome, allowing immediate sensing of increased calcium concentrations in the cell (152). This also allows the β cell to better control the rate and amount of calcium influx (152).

Without stimulation, syntaxin-1A is in a closed conformation (150). Syntaxin-1A in a closed form restricts the opening of calcium channel (150). This keeps the β cell repolarized. During priming, the v-SNARE synaptobrevin binds to the t-SNAREs SNAP25 and syntaxin-1A to form a trans-SNARE complex(74). The complex allows a conformational change in syntaxin-1A to an open form. In the open form, syntaxin-1A binds both the NBF1 and NBF2 of the potassium channel SUR1 subunits (74), losing the ability to restrict calcium channel closing yet inhibiting potassium channel opening. This keeps the β cell depolarized. The conformational change of syntaxin-1A, from closed to open, is considered the priming step (74). The increased calcium concentration in the cell, due to the increased ATP/ADP ratio, changes the trans-SNARE complex into a cis-SNARE complex. In order for the vesicle to fuse with the plasma membrane, the increased calcium concentration allows the disassociation of syncollin, previously associated with syntaxin-1A, from syntaxin-1A (74). Following fusion, the increased concentration of calcium in the cell stimulates exocytosis, or the release of insulin into the bloodstream (74).
1.5.6 The effects of increases in the guanosine triphosphate to guanosine diphosphate ratio

While ATP molecules are produced in the mitochondria, guanosine triphosphate (GTP) molecules are also produced. Increasing the GTP to guanosine diphosphate (GDP) ratio does not affect the $K_{\text{ATP}}$ channel or the calcium channel at the plasma membrane, but it does aid in the release of insulin (123). Cell division control protein 42 homolog (CDC42), a GTPase molecule, becomes activated by glucose stimulation in the cell (69). The surrounding GTP concentration plays a regulatory role in the activation of CDC42 (88, 89, 135). CDC42 along with other GTPases regulate actin filament remodeling and the translocation of insulin containing granules to gather at the plasma membrane (143). Meanwhile, activation of CDC42 stimulates the fusion of the vesicle to the plasma membrane (143). These processes occur in the second phase of insulin secretion. The GTP to GDP ratio affects both the docking and priming of insulin containing granules through the activation of ras-related protein 3A (RAB-3A) and ras-related protein 27 (RAB-27) (143), and also through the formation of exocyst complex (114) via ras-like GTPase family (RALA) (143). Therefore, GTP, in addition to ATP, plays a role in the secretion of insulin after stimulation with secretagogues such as glucose.

1.5.7 Biphasic insulin secretion

Insulin is secreted in two phases: the first and second phase (90). β cells have two pools of insulin granules: the reserve and readily releasable pools (52, 110). The reserve pool consists of vesicles that must be trafficked, docked, and primed prior to secretion (51). The readily releasable pool consists of granules docked and primed (7, 27). Mature granules, described
above, are sorted into the reserve pool in the β cell (7, 27). Some granules stay in this pool, and other granules are moved to the readily releasable pool following the five insulin secretion processes described above (55). The first phase of insulin normally lasts 5 to 10 minutes (143). This is where a chain of reactions occur after the stimulation by secretagogues allowing the readily releasable pool to be released immediately in order to quickly lower the blood glucose to maintain glucose homeostasis. This pool of granules is very small (less than 5 percent of the entire pool) (114), so the insulin secretion curve drops. During the drop in between first phase and second phase, insulin containing granules from the reserve pool are translocated to the membrane to refill the readily releasable pool of granules (124). The second phase of insulin secretion can be sustained for several hours depending on the blood glucose level (25, 110). It continues until the signals promoting secretion, such as high glucose, goes away. A defect in the first phase of insulin secretion is an early hallmark of type 2 diabetes (144), and a disruption in the second phase of insulin secretion is a later defect in type 2 diabetes (144).
Figure 1.3. The triggering pathway of insulin secretion in β cell. This demonstrates the steps of the glucose-stimulated insulin secretion pathway in sequential order: glucose entrance, glycolysis, pyruvate entry into the mitochondrion (pink), tricarboxylic acid (TCA) cycle, adenosine triphosphate (ATP) production by the electron transport chain (ETC), increase of the ATP/ADP ratio, closure of the ATP-sensitive potassium channel (KATP), depolarization, influx of calcium, reserve pool granules with insulin (yellow star) going through translocation, docking, priming, fusing, and lastly exocytosis.

1.6 Amplifying pathways with non-glucose secretagogues

The insulin secretion pathway described above is called the triggering pathway. There are many other pathways, called the amplifying pathways, that are stimulated by secretagogues other
than glucose to set off insulin secretion (53). As the name suggests, these pathways amplify the triggering pathway meaning the triggering pathway must be activated in order for the amplifying pathways to become functional (53). This protects the body from hyperinsulinemia and thus hypoglycemia as both the triggering and amplifying insulin secretion pathways are dependent on the glucose level present in the blood. The triggering pathway has been studied for decades and is accepted as the canonical secretion pathway (53). Meanwhile, the amplifying pathways are not fully known and thus not fully accepted. Non-glucose secretagogues include ions, fatty acids, and amino acids (55, 90).

1.6.1 Evidence for an amplifying pathway

In the triggering pathway, the increase of ATP to ADP closes the $K_{\text{ATP}}$ channel allowing the cell to depolarize resulting in the influx of calcium (53). If the potassium channel is inhibited from closing by the binding of diazoxide, then the cell can no longer depolarize, and thus is inhibited from secreting insulin (45). However, if diazoxide is added in combination with potassium to subsequently depolarize the cell, then increasing glucose can still stimulate increased insulin secretion independent of the closing of potassium channel (2, 123). Meanwhile, when sulphonylurea drugs are used to keep the potassium channels closed to maximally depolarize the cell, glucose can still further augment the insulin secretion level. Therefore, another pathway must exist that functions independent of potassium channels (53). When the glucose threshold for the triggering pathway and this second pathway were tested, glucose stimulated insulin secretion at a lower threshold in the triggering pathway than the other pathway. This indicates that this second pathway acts as an amplifying pathway to enhance the
function of the triggering pathway, but does not become active until after triggering has occurred (53).

1.6.2 Role of long-chain acyl-CoAs in insulin secretion

Free fatty acids have an important role in potentiating the amplifying pathway of glucose induced insulin secretion (22, 37). Previous studies have shown that insulin release is doubled when cells are incubated with free fatty acids prior to glucose stimulation (37). The action of free fatty acids is dependent on an esterification reaction to acyl-CoA by acyl-CoA synthase (ACS) to produce long-chain acyl-CoA (LC-acyl-CoA) (22, 37). LC-acyl-CoA activate the FFAR1/GPR40 receptor, a G-protein coupled receptor, resulting in increased intracellular Ca\(^{2+}\) through IP3 receptor signaling and activation of protein kinase D1 (PDK1) (41). In short, LC-acyl-CoA function as a signaling molecule to promote insulin release through regulating K\(_{\text{ATP}}\) channels (11), calcium channels (133) and promoting insulin exocytosis (32). Sub-cellular localization of LC-acyl-CoA is an important determinant of its action (22, 37). In the absence of glucose LC-acyl-CoA is rapidly transported into mitochondria through carnithine palmitoyl transferase 1 (CPT-1) where it is oxidized (22). Glucose stimulation promotes the production of malonyl-CoA, which directly inhibits CPT-1 to prevent oxidation of LC-acyl-CoA (109). The result of this is an increased cytoplasmic pool of LC-acyl-CoA that can further promote insulin secretion (109). However, a recent study has shown that glucose addition severely decreases malonyl-CoA production, which may suggest an alternative mechanism for the observed decrease in fatty acid oxidation (37). Interestingly, metabolomic analysis of glucose derived intermediates in the presence and absence of fatty acids showed that many LC-acyl-CoAs were esterified with glucose derived glycerol-3-phosphate (37). Together, fatty acids affect glucose
utilization by increasing glycolysis, the TCA cycle and lipogenesis, producing insulin secretion stimulating factors (see section 1.5) to potentiate glucose induced insulin secretion (37).

1.6.3 Amino acid derivatives

Other than fatty acids, amino acids such as alpha-ketoisocaproic acid (KIC) can also amplify insulin secretion (95). One line of studies has suggested that α-KG may be a metabolic coupling factor (104). KIC, which can be converted into α-KG, can promote insulin secretion (104). There are two pathways by which KIC and α-KG can be metabolized (Fig 1.4). KIC can be transaminated by branched-chain amino transferase (BCAT) in a reaction with glutamate that produces leucine and α-KG (155). Some have argued that α-KG itself must be a coupling factor, because mice with decreased BCAT activity shows lower insulin secretion (104). In contrast, others have argued that the leucine produced activates glutamate dehydrogenase (GDH), which in return catalyzes the deamination of glutamate to produce α-KG and ammonia (104). α-KG can also enter the mitochondria and the TCA cycle to augment insulin secretion (61).
Figure 1.4. Alpha-ketoisocaproic acid metabolism in relation to the insulin secretion pathway. $\alpha$-ketoisocaproic acid (KIC) enters the $\beta$ cell and into the mitochondrion where it can go through transamination with glutamate to become leucine. Glutamate, on the other hand, can be converted to alpha-ketoglutarate ($\alpha$-KG). The leucine produced by the transamination of KIC activates glutamate dehydrogenase (GDH) which catalyzes the deamination of glutamate to produce more $\alpha$-KG. $\alpha$-KG then feeds into the TCA cycle and continues in the insulin secretion pathway similar to the triggering pathway induced by glucose to enhance insulin secretion (61).

1.7 Benefit of mice as study tool

Mice and humans diverged more than 96 million years ago, but they maintained anatomical and genetic similarities (91). Anatomically, the organization and structure of mouse
tissues are similar to human tissues (91, 129). Genetically, the genomes of both mice and human have been sequenced and show 99 percent of their encoded genes are conserved (121). In addition, like in humans, mutations in mice can affect processes similar to human diseases such as altering risk of various cancers, heart disease, hypertension, diabetes, obesity, and metabolic and hormonal disorders (91, 129). These highlight the relevance of mice as a model to study human disease.

     In general, mice are good experimental model due to their availability, ease of breeding and housing, and their capability to speed up research. Obtaining human pancreatic tissue is impeded by the necessity for post-mortem access and consent for study (13). To increase the difficulties further, human pancreatic tissues may not come with reliable clinical information on the person. In addition, there is a large time delay from death until samples are available for use. On the other hand, mice are easily available from mouse breeding laboratories (121). Therefore, mice are good candidate to use in order to study human diseases.

     An additional utility are the tools available for genetic engineering of mice (121). The mouse genome can be directly manipulated to study human diseases (94) with the use of gene targeting technology such as gene knockout (129). This technology is commonly used in mice, and it has led Mario Capecchi, Martin Evans, and Oliver Smithies to win a Nobel Prize in 2007 for its usefulness (91). With the use of these technologies scientists have been able to implement basic science research to better understand the molecular basis of human disease. When using mice in studies, it is easy to keep all environmental factors constant and only change a single experimental factor of interest. This is very important as it is difficult to make conclusions from results influenced by many different factors at the same time. With the ease of working with mice and their similar biology to humans, it is also a great model to test different drugs prior to
use in humans. Overall, mice represent an excellent and flexible model that can be used as a tool to increase our knowledge in human diseases and to further develop therapeutics (91).

1.8 Classical inbred mouse strains versus wild-derived inbred mouse strains

1.8.1 Classical inbred mouse strains

Most research labs commonly use classical inbred mouse strains, such as B6, to study a disease including T2D (36, 127). An inbred strain is when all mice are genetically the same, so studying something in one strain is similar to studying something in a single person. This is beneficial because the results can be comparable when everyone uses the same strain. However, it removes the effect of genetic variation when studying disease.

The classical inbred mice originated from a small group of ancestors called fancy mice. In the early 20th century, these mice were kept as pets (26, 92). To create different fur coat colours (126, 127), mice from different places were crossed among each other by pet dealers and collectors. With only a handful of ancestors, classical inbred mice thus have limited genetic variation. They are composed of combination of three different subspecies with 90 percent of their genome from Mus musculus domesticus, and the remaining 10 percent consisting of Mus musculus musculus and Mus musculus castaneus (151). In fact, much less than half (25%) of the total genetic variation known in mice is found in these classical mouse strains (63, 85). In addition, the genetic variants in their genome are not uniformly distributed, consisting of regions where there is high variation between the strains and other regions with no variation (57). Therefore, the genetic studies that have been performed using these strains have missed testing much genetic variation for a role in disease.
1.8.2 Wild-derived inbred mouse strains

Because of the inter-relatedness of the classical inbred strains, wild-derived inbred mice were more recently created for research. Wild-derived mice are new inbred strains derived from mice caught in the wild in different locations. Thus they are unrelated to the classically studied strains and have increased genetic variation (36, 43, 67, 98, 126). Their increased genetic variation and many new variants they contain compared to any of the classical strains led them to be included in the Collaborative Cross and other large-scale genomics projects (18, 59, 63, 79). Genetically, wild-derived inbred mouse strains are derived from a single subspecies unlike the mosaic genetic pattern in classical inbred mice. Three common wild-derived inbred strains include PWD (PWD) from *Mus musculus musculus*, WSB (WSB) (97) from *Mus musculus domesticus*, and CAST/EiJ (CAST) from *Mus musculus castaneus* (43, 138, 139). Out of all known genetic variation in mice, 75 percent of these variants are found only in the wild-derived strains (36, 57, 63). These strains have a high level of genetic variation between each other and with the classical strains, that occurs throughout their genome.

Single nucleotide polymorphism (SNPs) of many different genes have been shown to increase the risk of T2D each by a small amount. It is thus thought that T2D results from the combined effect of many of these SNPs. Many studies of the classical inbred strains have mapped genes increasing risk of diabetes related traits, however few genes have been found and our understanding of these traits are still incomplete. Increasing the genetic variation studied in mouse models could help to identify new genetic factors. The reason is that more genetic variation increases the chance that a gene important for T2D contains a SNP significantly affecting its function and thus increases the possibility of more genes affecting T2D being identified. Since wild-derived inbred mice have increased genetic variation compared to the
classical inbred mice, the possibility of identifying genes associated with T2D that have never been tested increases (153). Since the classical strains do not show all aspects of human T2D development, the genetic variation in new strains may also result in different phenotypes that have not been found in the classical strains to study (19). This is why wild-derived mouse strains are a valuable addition to the original mouse model collection.

1.8.3 PWD wild-derived inbred mice

PWD mice are relatively new mouse strain, so a lack of studies prevents us from fully understanding this mouse strain. However, we have characterized the development of diabetes-related traits in PWD mice in our previous studies (73). In vivo studies were done on PWD mice fed with either rodent chow or a diet with a mixture high in fat (60%) and sugar (20%) (HFD) (73). At 4 weeks of age, chow fed PWD mice were observed to have increased fasting insulin relative to B6 mice, and more hyperinsulinemia when fed a HFD (73). PWD mice also secreted more insulin than B6 mice in our in vivo studies (73). Hyperinsulinemia may result from a compensatory effect for insulin resistance, but this is not the case for PWD mice. Young PWD mice are more sensitive to insulin, clearing glucose faster than B6 mice (73). A possible way PWD mice are prevented from hypoglycemia from this increase in insulin could be the increase of glucagon, which we have not yet addressed in our studies. They also remained leaner than B6 mice, even when fed with the HFD throughout 42 weeks of study (73). Only at 16 weeks of age do PWD mice fed with the HFD begin to develop obesity and decreased insulin sensitivity compared to chow fed PWD mice, whereas in HFD fed B6 mice obesity and insulin resistance were observed from 7 weeks of age (73). PWD mice have a later onset of obesity than B6 mice when fed with the HFD (73). By 10 months of age PWD mice became similar to B6 mice in their
level of insulin resistance and obesity, yet fasting insulin levels were still higher in PWD mice than in B6 mice (73). PWD mice had similar food intake and energy expenditure as B6 mice on either diet throughout the studies (73). In summary, PWD mice have hyperinsulinemia, and a robust early insulin response to an intraperitoneal glucose challenge prior to obesity and insulin resistance. PWD is a rare inbred mouse model as these mice exhibit hyperinsulinemia prior to insulin resistance.

1.8.4 WSB wild-derived inbred mice

The WSB mouse strain is a less studied strain, similar to PWD mice. In our previous studies, we have characterized WSB mice by feeding them with either chow or a HFD (73). These studies found that WSB mice fed with a HFD remained as lean as their chow fed siblings throughout 42 weeks of study (73). They were also leaner than B6 mice (73). WSB mice had increased food intake and exhibited less physical activity relative to B6 mice, yet WSB mice were resistant to T2D while B6 developed pre-diabetes (glucose intolerance) at an early age (73). This is likely due to their increased metabolic energy expenditure.

Consumption of a HFD is normally associated with increased insulin that was not seen in WSB mice (147). WSB mice maintained low fasting insulin levels, which could be explained by their high insulin sensitivity (73). However, they also secreted a much smaller amount of insulin in response to a glucose challenge in vivo (73). Furthermore, as high fasting insulin may increase the risk of insulin resistance, the low insulin levels in WSB mice may help protect them from developing insulin resistance (105).
1.9 The goals of this thesis

The processes affecting T2D are not fully elucidated. β cell mass and function are important factors in T2D development. The new wild-derived mouse models, PWD and WSB, were the same as B6 except their genes, so their phenotypic differences are due to genetic factors. The goal of my studies was to understand the physiological basis of their altered insulin levels. We hypothesized that PWD and WSB mice have altered β cells in structure and/or function, elevating or reducing their insulin level respectively.
Chapter 2: Methods

2.1 Animals

Male B6, WSB, and PWD mice were housed in the Centre for Disease Modeling. This is an environmentally controlled facility with twelve hour light/dark cycles (7am – 7pm). The mice were fed either a standard rodent chow (LabDiet 5010, Jamieson’s Pet Food Distributors, Delta, BC, Canada) or a HFD containing sixty percent calories from fat (lard) and twenty percent calories from sugar (sucrose and maltodextrin, D12492, Research Diets, New Brunswick, NJ) provided from weaning. The environment and the diets are the same as for our previous studies for characterizing wild-derived mouse strains in in vivo studies (73). The mice had unlimited water access. The mice were euthanized without fasting at the specified age by CO₂ asphyxiation followed by cervical dislocation. All procedures were done according to Canadian Council on Animal Care guidelines and approved by the UBC Committee on Animal Care.

2.2 Pancreatic islet isolation, static incubation, and perifusion

Islets were isolated from B6, PWD, and WSB mice by collagenase digestion (35) and purified using a modified filtration method by Salvalaggio et al (116). All islets were isolated from chow fed mice at 6 to 7 weeks of age to avoid complications due to changes occurring in response to obesity or insulin resistance. Immediately following euthanasia, a warm (37°C) solution of 1000 unit per mL collagenase in Hanks buffer (137 mM NaCl, 10 mM Hepes, 1 mM MgCl₂, 5.4 mM KCl, 4.2 mM NaH₂PO₄, 4.1 mM KH₂PO₄, 5 mM glucose) was perfused through the common bile duct after clamping the sphincter of Oddi. The pancreas was then removed, and placed in a tube containing the same collagenase solution. This tube was placed into a 37°C
water bath until the pancreas was no longer clumped. After digestion, the tube was filled with Hanks solution, shaken and centrifuged. This step was repeated three times to wash the digested pancreas. The digested material was then filtered through a 70 μm cell strainer into a petri dish containing RPMI media (with 11 mM glucose Invitrogen, Burlington, ON, Canada; including 1% penicillin and streptomycin mixture, and 10% fetal bovine serum) after the third wash. The islets were subsequently handpicked and incubated in the 11 mM glucose-containing RPMI media described above overnight at 37°C with 5% CO₂.

For perifusion, the next day one hundred size matched islets from each B6, PWD, and WSB mouse were picked into individual chambers for perifusion, as previously described (9, 99). Perfusate solutions were comprised of 3 mM glucose (basal), 20 mM glucose, or basal glucose containing 30 mM KCl, in Krebs Ringer Bicarbonate buffer (KRB) (118.41 mM NaCl, 4.69 mM KCl, 2.52 mM CaCl₂, 1.18 mM MgSO₄, 1.18 mM KH₂PO₄, 25 mM NaHCO₃, 5 mM HEPES, 0.5% BSA), and were flowed through the chambers at a rate of 1 mL per minute for 20-30 minutes for each secretagogue. Perfusion of the various secretagogues were interrupted with 30 minutes basal glucose in between. Perfusate fractions (1.7 mL) were collected every 5 minutes and stored at -20°C until analysis. Insulin was measured by enzyme-linked immunosorbent assay (ELISA), as described and also see section 2.4 (64). These studies were performed in collaboration with Dr. Xiaoke (Betty) Hu in the laboratory of Dr. James Johnson. Dr. Hu and I worked as a team with her isolating the islets and performing the perifusion while I picked the islets and ran the ELISA.

For static incubation, on the day of the experiments, the islets were handpicked and incubated in the specified buffers in glass tubes with mesh bottoms within larger glass tubes (104). Initially, following picking, 5 size matched islets were placed in each tube with 1.7 mM
glucose-containing KRB for a 45 minute pre-incubation period. Afterwards, the islets in the baskets were transferred into different glass tubes containing the test solution. The test buffers used in these studies were as follows: 1.7 mM (low) and 16.7 mM (high) glucose as controls, plus the experimental conditions (2-3 per experiment). To test the glucose sensitivity, the experiments included 5.5 mM glucose and 11 mM glucose as experimental conditions. Other secretagogues (Table 2.1) were tested in KRB buffer containing 1.7 mM glucose plus either: 15 mM KIC, 30 mM KCl, 0.25 mM diazoxide and 30 mM KCl, or 0.25 mM diazoxide. Additional studies were performed with KRB buffer containing 16.7 mM glucose and either 0.5 mM palmitate, 30 mM KCl, 0.25 mM diazoxide and 30 mM KCl, or 0.25 mM diazoxide. For 30 mM KCl, the salt concentration in the buffer was adjusted by reducing the sodium chloride concentration in the KRB (to 88.41 mM) in order to prevent hypertonic effects. For 0.5 mM palmitate, 5.6 mg palmitate was first dissolved in 1 mL H2O and 10 ul 1 N NaOH at 70°C, then 0.5 mL of this solution was added to a mixture of 0.66 mL 20% BSA and 18.84 mL of KRB with either low or high glucose solution (54). Following incubation in these test buffers at 37°C for 45 minutes, the baskets containing the islets were transferred into final glass tubes containing acid ethanol (21 mL H2O, 75 mL 100% ethanol, and 15 mL concentrated HCl) and stored at -20°C. The media was stored at -20°C until measurement of insulin. Each experimental condition was performed in triplicate in each experiment, and each replicate was comprised of 5 islets. The experiments were repeated multiple times, and the number of animals used for each condition is specified in each figure legend.
Table 2.1. The secretagogue cocktails. Different concentrations of different secretagogues mixed with either 1.7mM low glucose or 16.7mM high glucose. KIC, α-ketoisocaproic acid. KCl, potassium chloride. DIA, diazoxide. PA, palmitate. +, indicated nutrients combined; -, indicated nutrients not combined.

<table>
<thead>
<tr>
<th></th>
<th>15mM KIC</th>
<th>30mM KCl</th>
<th>0.25mM DIA</th>
<th>0.25mM DIA &amp; 30mM KCl</th>
<th>0.5mM PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7mM Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>16.7mM Glucose</td>
<td>-</td>
<td>+</td>
<td>+</td>
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After a minimum of 24 hours in acid ethanol at -20°C, the islet lysates from the static incubation were diluted 100 times in 1X phosphate buffered saline (PBS) containing 1% BSA and neutralized with 10 N NaOH. Both diluted islets and media (no dilution) were assayed for insulin by ELISA (as in section 2.4). The data were expressed as follows: total secreted insulin per islet in 45 minutes incubation, insulin content per islet, and fractional insulin referring to total secreted insulin over secreted insulin plus insulin content.

2.3 Pancreatic insulin content

Following euthanasia, pancreata from B6, PWD, and WSB mice were dissected, weighed and rapidly placed in 4 mL ice-cold acid ethanol. Pancreata were minced manually for 4 minutes and stored at -20°C for a minimum of 18 hours. The next day, minced tissues were neutralized with sodium hydroxide and diluted 10,000 times with 1X PBS 1% BSA as previously described (20). Insulin was measured using the ELISA described (see section 2.4).
2.4 Enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Corning, costar #3690; 96-well, ½ area) were coated with 3 ug/mL monoclonal antibody D6C4 (Research diagnostics insulin antibody clone) in 1X PBS overnight covered at room temperature. The next day, the plates were emptied and incubated with 1X PBS containing 4% RIA grade BSA (sigma A-7888) for an hour covered at room temperature. After the plates were emptied, 25 ul insulin standards and samples were added into the wells in duplicates. 25 ul of the biotinylated anti-insulin detecting antibody D3E7 (Research diagnostics, insulin antibody clone) diluted 5000 times with 1X PBS 1% BSA was added directly to the samples and standards containing wells and incubated for an hour at room temperature. The plates were then emptied and washed 3 times with wash buffer (5 mM Tris with 0.2% Tween 20 at pH 8.0) followed by the addition of 50 ul streptavidin-HRP (Pierce 21126) diluted 1000 times with 1X PBS 0.1% BSA, and the plates were incubated for 30 minutes at room temperature. 1 tablet of OPD (o-phenylenediamine, Sigma P-5412) was dissolved in 12 mL citrate phosphate buffer (17.9 mM Na₂HPO₄•7H₂O and 49 mM anhydrous citric acid adjusted to pH 5 with NaOH, then 0.03% H₂O₂ was added and the total volume brought to 1000 mL with filtered water) and pipetted into each well (50 uL) after the plates were emptied and washed 3 times with wash buffer. The plates were incubated at room temperature until the highest insulin standard became dark. Stop solution (0.18 M sulphuric acid) was then added to quench the reaction and prevent further colour development. These procedures were previously described (64). Colour development was measured using a spectrophotometer (Infinite M1000 microplate reader, Tecan, Durham, NC, U.S.A) with reference wavelength 620 nm and measurement wavelength 492 nm. A standard curve was constructed and fit with a cubic spline. Sample concentrations were interpolated.
2.5 Immunohistochemistry

The mice were divided into six groups: B6, PWD, and WSB fed on either chow or HFD. Each wild-derived strain was compared to the same group of B6 for immunohistochemistry. The pancreata of each group were collected at the specified age, weighed, fixed overnight in 4% paraformaldehyde (in PBS) at 4°C, infiltrated overnight in 30% sucrose (in 1X PBS) at 4°C, and frozen in Neg50 media (Richard Allan Scientific, Canada). In our preparation processes, the pancreases were folded over, such that each section contained a portion of the entire pancreas (such as both head and tail of the pancreas). The sections of the pancreas were also spaced far apart to minimize repeated measurement of the same islet. Ten micron cryosections (each a minimum of 300 microns apart) were obtained at multiple levels through the pancreas for immunofluorescent staining as previously described (20). This helped to prevent biased measurement of the unevenly distributed islets within the pancreas (118). Sections were stored at -20°C until staining. For each mouse, 2 to 3 ten micron cryosections from different positions were stained and analyzed, with a replicate of 5 mice per strain and per diet. Due to the preparation as mentioned, each section contained more than one area of the pancreas.

Sections were first blocked with 10% donkey serum (Jackson ImmunoResearch) in 1X PBS for 30 minutes at room temperature in a humid chamber. After 2 washes with 1X PBS, the sections were stained with guinea pig-anti-insulin antibody (Sigma I-8510, 1:500) and either rat-anti-PECAM antibody (1:50) or mouse-anti-glucagon antibody (Sigma G-2654, 1:100) in 5% donkey serum as primary antibodies for 2 hours at room temperature in humid chamber. The slides were then washed in 0.05% Tween 20-1X PBS three times for 5 minutes each time. Donkey-anti-guinea pig-Cy3 antibody (Jackson ImmunoResearch 706-165-148, 1:500) and either goat anti-rat IgG-FITC antibody or donkey-anti-mouse-FITC antibody (Jackson
ImmunoResearch 715-095-150, 1:200) in 10% donkey serum were used as secondary antibodies and incubated for 30 minutes at room temperature in a humid chamber. Sections were washed 5 times in 0.05% Tween20-1X PBS followed by filtered water and then counterstained with DAPI, coverslipped and stored in -20°C until imaging. Stained tissues were imaged on a fluorescent microscope equipped with a motorized stage for automated scanning (Zeiss Axiovert 100). Scanned images were reassembled and insulin, either endothelial cell or glucagon, and total tissue staining areas were measured using Slidebook Software (Intelligent Imaging Innovations, Denver, Co, USA).

2.6 Statistical analysis

Statistical analysis was performed by t-test when comparing 2 groups, ANOVA with post-hoc Tukey pairwise comparisons, or two-way ANOVA (islet size distributions) followed by Bonferroni pairwise comparisons between the genotypes in each size group, as appropriate. Data are shown as mean ± standard error. P-values < 0.05 were considered significant.
Chapter 3: Mechanisms that may contribute to the high fasting insulin in PWD mice

3.1 Results

3.1.1 Insulin secretion dynamics in PWD and B6 mice

We have previously shown that young PWD mice have increased insulin secretion in response to an intraperitoneal glucose challenge in vivo (73). We hypothesized that the insulin phenotype of PWD is due to changes in the insulin secretory function of islets. To test this hypothesis, we isolated islets from young PWD and B6 mice and performed perifusion studies (Fig 3.1). Through the perifusion process, we examined whether secretion was different under low glucose conditions, the time they began to respond to secretagogues, the amount of their insulin secretion, and the pattern of their secretion.

When islets were incubated with basal low glucose (3mM), the islets of both mouse strains, B6 and PWD, secreted low levels of insulin (Fig 3.1A). Media from PWD islets contained an average of 0.15 ng/mL of insulin, which is not different than the average insulin released from B6 islets (0.17 ng/mL). This is important because if PWD islets were secreting more insulin than B6 islets, then PWD islets may have been leaky to start off with or may have had unregulated higher constitutive secretion. We also saw a constantly similar low secretion pattern whenever the islets were perfused with basal glucose throughout the experiment (Fig 3.1A).

When stimulated with high glucose (20 mM glucose), B6 islets displayed increased insulin secretion compared to levels in low glucose (Fig 3.1A). Secretion from B6 islets followed the expected biphasic pattern: the rapid first phase followed by a slower second phase. This is
more evidence that collected islets were healthy and functioning properly. The first phase in our experiment lasted for the first 10 minutes (Fig 3.1A, min 30 to 40). The second phase that came after lasted for at least 20 minutes (Fig 3.1 A, min 40 to 60) (51). Islets from PWD mice showed a markedly increased secretion in response to high glucose compared to islets from B6 mice (p = (Fig 3.1A, min 30 to 60). Notably, the two phases were not clearly distinguished. There was a consistently high insulin secretion across all time points, without a substantial drop in secretion from min 30 to 60. Total area under the curve (AUC) from the average basal glucose level (min 30 to 60) was calculated in order to determine the total amount of insulin secreted by islets of both mouse strains in response to high glucose. B6 islets secreted a total of 1.16 ng/mL insulin after high glucose stimulation whereas PWD islets secreted a total of 5.36 ng/mL, 4.6 times higher than B6 islets (Fig 3.1B, p < 0.0001).

At 60 minutes into the perifusion experiment, the buffer containing high glucose was replaced by buffer with basal glucose. This immediately caused a drop in the insulin secretion for both mouse strains (Fig 3.1A, min 65 to 85). This again showed that the glucose-stimulated insulin secretion of these islets was regulated. As observed in the initial stage, insulin secretion of PWD mice was similar to that of B6 mice.

As described (section 1.5.4) closure of the $K_{ATP}$ channels causes cell depolarization and results in insulin secretion. This is a critical step in the insulin secretion pathway. Cell depolarization can be induced in vitro by incubating the islets in high concentrations of extracellular potassium (30). To determine if the factor(s) affecting high insulin secretion in PWD mice in in vivo studies lies downstream of this critical step, we then perfused the islets with 30 mM potassium in basal glucose (Fig 3.1A, 90 to 105 minutes). The potassium added into the
experiment maximally depolarizes the cells, as if all the potassium channels are closed (30, 42, 46).

In response to 30 mM potassium, insulin secretion was increased from B6 islets compared to that induced by high glucose. Again, the biphasic pattern was visible in B6 mice with a rapid phase at min 90 to 95 and a slower second phase at min 95 to 105. Meanwhile, PWD islets had similarly high insulin secretion as when stimulated with high glucose. This time, however, PWD islets showed 2 phases with a rapid first phase similar in magnitude to B6 islets at min 90-95, and then a drop followed by a plateau of insulin secretion at min 95-105, higher than the second phase of B6. When the AUC was calculated, B6 secreted a total of 2.38 ng/mL of insulin whereas PWD islets secreted 4.22 ng/mL (1.7 times more than B6, p = 0.02, Fig 3.1C). The difference between B6 and PWD islets was much larger in response to glucose than to potassium. This suggested that multiple factors may affect the insulin phenotype of PWD mice, with a major one affecting their response to glucose and a smaller component affecting steps downstream of depolarization.

Following incubation with potassium, the islets were returned to basal glucose concentrations (Fig 3.1A, min 110 to 130). We saw again that collected islets returned to their low insulin secretion similar to their previous basal glucose response. This indicated that the islets were still healthy and responsive.
Figure 3.1. Insulin secretion studies between PWD and B6. Thin line/black bar B6, thick line/white bar PWD. A, Perifusion studies of islets stimulated with 3 mM basal glucose, 20 mM glucose, and 30 mM potassium chloride in basal glucose. Buffers were changed at the times indicated. The y-axis shows the insulin concentration secreted into the 1.7 mL collected media in each 5 min period (ng/mL). B, Total area under the curve in the perifusion studies during stimulation with 20 mM glucose (30 min to 60 min) from basal levels. C, Total area under the curve of perifusion studies during stimulation with 30mM potassium chloride. *p<0.05, **p<0.01, ***p<0.001 B6 vs. PWD islets incubated with the same secretagogue. (n=10 B6 mice, n=8 PWD mice).
3.1.2 Insulin secretion in response to a range of glucose concentrations

To confirm the above findings and examine the response of PWD islets to various secretagogues, static incubations were performed (Fig 3.2). To examine the glucose responsiveness of the islets, a range of glucose concentrations was used: 1.7 mM, 5.5 mM, 11 mM, and 16.7 mM. B6 islets secreted insulin in response to all glucose concentrations above 5.5 mM (Fig 3.2A). Similarly, PWD islets also responded to glucose concentrations above 5.5 mM. In mouse islets, physiological elevations of plasma glucose concentrations above approximately 6 mM trigger detectable insulin secretion (111). Consistent with the above (Fig 3.1), we found islets from PWD mice were not different in insulin secretion compared to islets from B6 mice in both 1.7 mM and 5.5 mM glucose. This result further supports the perifusion findings that PWD mice do not have more basal insulin secretion than B6 mice.

Consistent with the perifusion results (Fig 3.1), we found islets from PWD mice had significantly increased insulin secretion when stimulated with high glucose concentrations compared to B6 mice. PWD mice secreted 40% more insulin at 11 mM (sub-maximal) glucose concentrations (p<0.01) and ~2 times more insulin at 16.7 mM glucose (p<0.001) relative to B6 mice.

To examine whether the increased secretion might result from increased insulin in the islets, we measured islet insulin content. Surprisingly, the insulin content of the islets from PWD mice was significantly lower than that in islets from B6 mice (Fig 3.2B). Therefore when insulin secretion was expressed as a percentage of the total islet insulin content, PWD mice secreted fractionally much more of their insulin in response to glucose compared to B6 mice at all glucose concentrations (Fig 3.2C). However, our goal was to understand why PWD mice have high insulin levels in their blood, which are affected by the total amount of insulin that is released.
from their islets, not the proportion of what they contained. Thus, we focused our analysis on the total insulin secretion data for the remaining static incubation experiments.

Figure 3.2. Insulin secretion in response to increasing glucose concentrations in PWD and B6 islets. Black bars represent B6 and white bars represent PWD. A, total insulin secretion in response to increasing glucose concentrations. B, Mean insulin content per islet in each glucose concentration. C, Fractional insulin secretion to increasing glucose. *p<0.05,**p<0.01,***p<0.001 B6 vs PWD islets incubated in same glucose concentration. +p<0.05,++p<0.01, +++p<0.001 vs. 1.7mM glucose from the same strain. ^p<0.05, ^^p<0.01, ^^^p<0.001 vs. 5.5mM glucose in the same strain. oo p<0.01, ooo p<0.001 vs. 11mM glucose in the same strain. (n=12 for B6, n=9 PWD).
3.1.3 Insulin secretion in response to other nutrient secretagogues

Glucose-stimulated insulin secretion involves several steps (section 1.5). Any of these steps may affect the insulin phenotype of PWD mice. Metabolism is an important step, and other metabolites can promote insulin secretion, entering the pathway at different steps. Therefore, other nutrient secretagogues were examined in order to determine whether factors in metabolism could account for the difference in insulin secretion between B6 and PWD mice.

Palmitate, a fatty acid, is known to potentiate insulin secretion (33, 37, 47). We stimulated both B6 and PWD islets with a mixture of high glucose (16.7 mM) and palmitate (0.5 mM) (Fig 3.3). Palmitate potentiated the insulin secreted of B6 islets by two-fold relative to their response to high glucose alone (p < 0.0001). Similar results were observed for PWD islets, where insulin secretion was increased beyond the high level already seen in response to high glucose alone (Fig 3.3, p < 0.0001). PWD mice secreted 41% more insulin than B6 mice when stimulated with palmitate (p = 0.01). This indicated that glucose is not the sole compound capable of stimulating increased insulin secretion from PWD mice. The difference in the total amount of insulin secreted between B6 and PWD mice was larger when palmitate was added to high glucose (0.27 ng/mL) than with high glucose alone (0.16 ng/mL), suggesting the difference was not due to the effects of high glucose alone. These data suggest that a difference between the strains lies downstream of β-cell glucose sensing and uptake.
Figure 3.3. Insulin secretion of B6 and PWD islets stimulated by palmitate. A, Insulin secretion of islets incubated with 0.5 mM palmitate in 16.7 mM glucose and 16.7 mM glucose alone (n=19 for B6 (black), n=13 PWD (white)). *p<0.05,**p<0.01,***p<0.001 B6 vs PWD islets incubated in same secretagogue. +p<0.05,++p<0.01, +++p<0.001 vs. the same strain in 1.7mM glucose. ooo p<0.001 vs. the same strain in 16.7 mM glucose.

In order to identify where in metabolism the difference in PWD mice may be, KIC, that enters pathway through the TCA cycle, was used (Section 1.6.3). Both B6 and PWD islets secreted a similar amount of insulin when stimulated with a mixture of low glucose (1.7 mM) and KIC (15 mM) as with high glucose (16.7 mM) alone. PWD islets stimulated with KIC and low glucose secreted 6 times more insulin than low glucose alone and 2.5 times more insulin than B6 islets (Fig 3.4, p < 0.001). The difference in the amount of insulin secretion between the
two strains was maintained when the islets were stimulated by KIC. This provided further support that the insulin phenotype seen in PWD mice was unrelated to differences in glycolysis. Instead these data suggested that the factor(s) in the insulin secretion pathway affecting PWD mice is(are) located downstream of the generation of α-KG in the TCA cycle.

**Figure 3.4. Insulin secretion stimulated by KIC.** Islets from B6 and PWD mice were incubated with 15 mM α-ketoisocaproate (KIC) in 1.7 mM glucose or either 1.7 mM or 16.7 mM (as a positive control) glucose alone. (n=10 for B6 (black), n=9 PWD (white)). *p<0.05,**p<0.01,***p<0.001 B6 vs PWD islets incubated in same secretagogue. *p<0.05,**p<0.01,***p<0.001 vs. the same strain in 1.7 mM glucose.

### 3.1.4 Insulin secretion from PWD islets in response to non-metabolizable secretagogues

To determine whether the strain difference is upstream or downstream of the closure of the K$_{ATP}$ channel, we next tested the effects of potassium (Fig 3.5). Increased extracellular potassium triggers depolarization of β cells.
Figure 3.5. Insulin secretion of PWD and B6 islets stimulated by non-metabolized secretagogues. Black low glucose B6, white low glucose PWD, black with white stripe high glucose and secretagogue B6, white with black stripe high glucose and secretagogue PWD. Islets incubated with 30 mM KCl and/or 0.25 mM diazoxide (DIA) with 1.7 mM or 16.7 mM glucose. (n=24 for B6, n=28 PWD). The data suggest that altered factors may be present upstream and downstream of the depolarization step in the insulin secretion pathway. Additionally, the altered factors are likely to be in the triggering pathway and not in the amplifying pathway. *p<0.05,**p<0.01,***p<0.001 B6 vs PWD islets incubated in same secretagogue. *p<0.05,**p<0.01,***p<0.001 vs. the same strain in 1.7 mM glucose. **p<0.001 vs. same strain in 16.7 mM glucose. +++p<0.001 vs. same strain in 1.7 mM glucose and 30 mM potassium. "p<0.05, "p<0.01, ""p<0.001 vs. same strain in 1.7 mM glucose, 30 mM potassium, and 0.25 mM diazoxide. bbbp<0.001 vs. same strain in 16.7 mM glucose with 0.25 mM diazoxide.
When the islets were incubated with potassium (30 mM in the presence of low glucose (1.7 mM)), insulin secretion was stimulated in both strains. In contrast to the ~2-fold difference in glucose-stimulated insulin secretion in between the strains, a small but significant increase (35%) in insulin secretion from PWD islets compared to B6 islets in response to potassium was seen (Fig 3.6, 3rd group). This is consistent within the perifusion studies, in which PWD islets secreted about 50% more insulin in response to potassium than B6 islets (Fig 3.1A). These data suggested that factor(s) downstream of depolarization might contribute in part to the increased insulin secretion, although the majority of the difference results from steps upstream.

The amplifying pathway was identified through the observation that even when cells are depolarized, secretion is increased in high glucose compared to low glucose (52). We examined whether there were strain differences in the amplifying pathway by comparing the previous results to those when we added 30 mM potassium into high glucose (16.7 mM) compared to low glucose. The addition of high glucose allowed both the triggering and the amplifying pathway to be activated at the same time (52). If the strain difference was due only to factors downstream of depolarization (e.g. granule trafficking, insulin content, etc.) we hypothesized that it would still be evident if potassium was added to stimulation by high glucose. The addition of potassium to high glucose-containing buffer overcame the strain difference in response to glucose-stimulated insulin secretion (Fig 3.6, 3rd group). This suggested that the main strain difference lies upstream of potassium depolarization, and that there was no difference in the generation of amplifying factors between the two strains (Fig 3.6, 4th group). This suggested that the majority of the difference between the strains is due to factor(s) upstream of the depolarization, whereas a more minor difference that can be overcome by amplifying signals may lie downstream of this step.
To further examine the role of the ATP-sensitive potassium channel (K\textsubscript{ATP}) in these strain differences, diazoxide was used (Fig 3.5). Diazoxide prevents closure of these potassium channels, preventing depolarization and opening of the voltage-gated calcium ion channels, resulting in the inhibition of insulin secretion (36, 39, 62, 88, 92). With the presence of diazoxide, the insulin secretion to high glucose (16.7 mM) was inhibited (Fig 3.6, 2\textsuperscript{nd} group). This suggested that the secretion depended on the closure of the K\textsubscript{ATP} channel and not other channels or pathways bypassing this channel.

Next, we examined glucose-stimulated insulin secretion under conditions in which alterations in the potassium channel are removed. To do this, we inhibited the potassium channel closure with diazoxide but then caused depolarization with extracellular potassium. Under these conditions, there was no difference between the strains (Fig 3.6, 4\textsuperscript{th} group). That removing the activity of the K\textsubscript{ATP} channel overcomes the strain difference suggests that the major strain difference lies in processes affecting the closure of this channel. Altogether, the static incubation results suggested that the primary factor(s) affecting the increased insulin secretion from PWD islets is located in the generation of triggering signals prior to the depolarization process, for example in the production of ATP.
3.1.5 Beta cell morphology and pancreatic insulin content

We also examined whether there are factors that could also be affecting the fasting insulin levels of PWD mice other than insulin secretion. Increased insulin levels could also result
from changes in β-cell mass or insulin content such as more islets, bigger islets, or more insulin per islet.

We begun our examination with pancreatic sections collected at twenty weeks of age. By this age, both B6 and PWD mice on HFD had developed insulin resistance (73). The islet structures in both strains looked normal, with glucagon staining area around the insulin staining area in the center (Fig 3.7A). The islet size distribution was similar between the two strains for chow fed mice (Fig 3.7B). In mice fed the HFD, B6 mice had a reduction in the relative number small islets with a corresponding increase in medium sized islets. This tendency towards increased islet sizes was seen to a lesser degree in HFD-fed PWD mice, which had a similar size distribution to the chow-fed PWD mice. However, this did not lead to any difference in mean islet size between both strains on either diet (Fig 3.7C), or the percentage of pancreatic area stained for insulin (Fig 3.7D). The pancreas weights for PWD mice were significantly smaller than those of B6 mice for both diets (Fig 3.7E). Therefore, the total β cell mass in PWD mice on either diet tended to be reduced compared to B6 mice, although the differences were not significant (Fig 3.7F). The glucagon-staining area per pancreatic area tended to be a bit higher in PWD mice (Fig 3.7G), such that total α cell masses were similar between PWD and B6 mice (Fig 3.7H). Therefore, we conclude that the increased fasting insulin and insulin secretion seen in adult PWD mice relative to B6 in previous studies (73) were not due to structural differences in their islets, size differences in their islets, or augmented β cell mass.
Figure 3.7. Islet architecture and morphometry at twenty weeks of age in PWD and B6 mice. A.

Immunofluorescent staining of representative islets from B6 and PWD mice. Blue nucleus (DAPI), red insulin, green glucagon. B, Islet size distribution. Black B6, white PWD, solid colour chow diet, striped HFD. C, Mean islet size. D, Percentage of pancreatic tissue (DAPI-staining) area stained positively for insulin. E, Pancreas weight. F, β cell mass. G, Percentage of pancreatic tissue (DAPI-staining) area stained positively for glucagon. H, α cell mass. *p<0.05, ***p<0.001 B6 vs. PWD mice consuming the same diet. **p<0.01, +++p<0.001 Chow vs. HFD fed of the same strain. (n=5 mice per strain per diet).
The pancreatic sections collected at twenty weeks of age were from mice that were fed a HFD started from weaning and had become insulin resistant. This could have promoted compensatory changes in their islet mass that masked an initial difference between the strains. This was supported by the change in islet sizes in B6 mice fed with chow versus the HFD (Fig 3.8). Therefore, we examined younger mice to determine whether increased β cell mass could have contributed to the increased insulin levels in young PWD mice. We examined pancreatic sections obtained from mice at four weeks of age, after only one week of the HFD. At this age, neither B6 nor PWD mice had developed diet-induced obesity or reduced insulin sensitivity (73).

As expected, the islets appeared normal at this age, with a core of insulin staining surrounded by glucagon staining cells in the periphery (Fig. 3.8A). The distribution of islet sizes between B6 and PWD mice was not different in chow fed mice, whereas PWD mice had a slightly higher proportion of large islets than B6 mice fed the HFD (Fig 3.8B). Thus, the mean islet size of PWD mice consuming the HFD was significantly increased compared to B6 mice consuming the HFD (Fig 3.8C). However, when the pancreas weight was measured, it was found that PWD mice fed the HFD had a significantly smaller pancreas (Fig 3.8D). Similar trends were observed in the chow-fed mice, although these were not statistically significant. The percentage of their pancreatic area stained with insulin was similar between both strains and for both diets (Fig 3.8E). The finding that PWD mice had similar total staining areas as B6 mice yet had a trend towards larger individual islets suggested that PWD mice may have a smaller number of islets than B6. As they had a similar insulin staining area but a smaller pancreas, total β cell mass for mice consuming both diets tended to be lower in PWD mice than in B6 mice (Fig 3.8F). Therefore, we concluded that the higher fasting insulin phenotype from in vivo studies in PWD
mice relative to B6 (73) was not likely due to either a deviation in structure of their islets or an increase in β cell mass.

α cells are located inside the pancreatic islets and produce the counter regulatory hormone glucagon. Because PWD mice had higher insulin levels, but normal glucose levels, we hypothesized that glucagon levels are elevated in PWD mice. To determine whether PWD mice have increased glucagon-producing α cells, we examined α cell mass in the same sections. No significant differences in their pancreatic area stained for glucagon or total α cell mass were found between both strains and diets (Fig 3.8G, H).
Figure 3.8. Islet architecture and morphometry at four weeks of age in PWD and B6 mice. A, Immunofluorescent staining of representative islets from B6 and PWD mice. Blue DNA (DAPI), red insulin, green glucagon. B, Islet size distributions. Black B6, white PWD, solid colour chow diet, striped HFD. C, Mean islet sizes. D, Pancreas weights. E, Percentage of pancreatic tissue (DAPI-staining) area stained positively for insulin. F, β cell mass. G, Percentage of pancreatic tissue (DAPI-staining) area stained positively for glucagon. H, α cell mass. *p<0.05,**p<0.01,***p<0.001 B6 vs PWD mice consuming the same diet. **p<0.01 Chow vs. HFD fed of the same strain. (n=5 per strain per diet).
As the prior data were based on staining areas which do not measure the amount of insulin present, we directly measured pancreatic insulin content in young mice. Consistent with the lack of difference in insulin staining area per tissue area, the total insulin per gram of pancreas between the two strains was similar (Fig 3.9A), suggesting a similar insulin content per islet or per β cell. However, as observed previously (Fig. 3.7, 3.8), the pancreata of PWD mice on both diets were significantly smaller than those of B6 mice (Fig 3.9B). Thus the total pancreatic insulin content of PWD mice for both diets were significantly lower than in B6 mice fed the corresponding diet (Fig 3.9C). Therefore, increased total pancreatic insulin content does not apparently account for the increased insulin levels between PWD and B6 mice.
Figure 3.9. Total pancreatic insulin content. A, whole pancreas insulin content per gram of pancreas. B, Pancreas weight. C, Total pancreas insulin. Black B6, white PWD, solid colour chow diet, striped high fat diet. *p<0.05,**p<0.01,***p<0.001 B6 vs PWD mice consuming the same diet.++p<0.01 Chow vs. HFD fed of the same strain. (n=10 for both diets in B6, n=11 for chow and n=9 for high fat diet in PWD).
3.2 Discussion

In previous studies, lean PWD mice showed fasting hyperinsulinemia without insulin resistance at a young age (73). In these studies we examined potential contributors to this hyperinsulinemia. Hyperinsulinemia can occur when the body becomes resistant to insulin. Since PWD mice were hyperinsulinemic compared to B6 mice at an age at which they were leaner and more insulin sensitive (73), we investigated the pancreatic islets as a primary cause of their hyperinsulinemia. In order to compare the islets between the two strains, size-matched islets from both PWD and B6 mice were used. We found that PWD islets secreted more insulin than B6 islets in response to increased glucose concentrations, along with other nutrient secretagogues, palmitate and KIC. The difference was consistent with our previous finding of increased insulin secretion in response to glucose in in vivo studies (73). We concluded that increased nutrient-stimulated secretion likely plays a role in the PWD insulin phenotype. The difference between the two strains was observed when the islets were stimulated with KIC which enters in the TCA cycle and which replicated the effect of glucose. From this we concluded that factor(s) downstream of the entrance of α-KG into the TCA cycle likely contribute to the hyperinsulinemia of PWD mice.

Processes downstream of the TCA cycle affecting insulin secretion include the electron transport chain to synthesize ATP and raise the ATP/ADP ratio, depolarization of the cell by the closure of potassium channels, calcium influx, and exocytosis (74, 114). A common way to examine the downstream processes of insulin secretion pathway is to stimulate the islets with potassium which maximally depolarizes the cells (30, 42). Potassium stimulated more insulin secretion from PWD islets relative to B6 islets in the perifusion with a similar trend in the static incubation. The difference between the strains was much smaller than that observed in response
to glucose. This suggested that a part of the difference between the strains was located downstream of the potassium depolarization process including calcium influx and exocytosis.

The difference in secretion in response to potassium in low glucose and in high glucose is known as the amplifying effect of glucose (53). The addition of potassium to high glucose overcame the difference between the two strains. This suggested that the strain difference was not in response to the generation of amplifying factors during metabolism. Furthermore, when the K\textsubscript{ATP} channel was bypassed by the addition of diazoxide and potassium (53), the strain difference in glucose stimulated insulin secretion was also abolished. This suggests that the difference between the strains may lie in triggering factors leading to the closure of the K\textsubscript{ATP} channel. Combined, these results suggested that the major factor affecting the increased insulin secretion in PWD islets lies upstream of depolarization process yet downstream of entry of α-KG into the TCA cycle, such as processes within the electron transport chain, perhaps in the generation of ATP. Therefore, overall, the insulin secretion studies suggested a major factor upstream of the potassium channel and possibly a minor factor downstream of this channel likely contribute to the increased insulin secretion and thus potentially the hyperinsulinemia of PWD mice relative to B6 mice.

PWD mice secreted more insulin than B6 mice, yet their islets contained less than half of the amount of insulin relative to B6 islets. Insulin is synthesized in the rough endoplasmic reticulum (RER), and stored inside the granules in the β cells (55). These granules are transported from the cytosol to the plasma membrane where the docking, priming, fusing, and exocytosis processes occur (55). During secretion, only a small percentage of granules are exocytosed (52, 110). There is also evidence that newly synthesized granules are preferentially trafficked for exocytosis (27, 142). Thus, several potential biological explanations for augmented
insulin secretion and lowered insulin content inside the pancreatic islets are possible. PWD islets, relative to B6 islets, could have a smaller amount of granules in the reserve pool than B6 islets, reducing their overall insulin content, but with PWD β cells having a higher number of granules docked and/or a higher efficiency in trafficking new granules to the membrane to increase their total secretion. Another possibility could be that both strains have similar granule trafficking efficiencies, but that PWD mice could have more insulin in each granule, again with fewer total granules in the cell than B6 mice. These potential explanations could be examined in the future by observing granule morphology through the use of electron microscopy.

The reason for the reduced insulin content in PWD islets compared to B6 islets is not clear. It is possible that they have reduced insulin synthesis or that they can not synthesize insulin fast enough to replace the amount secreted. This could be tested by pulse-chase analysis (107). Although we consistently measured a reduced insulin content in islets from PWD mice, there may also be a technical explanation for this finding. Both strains of islets were incubated overnight in RPMI media containing 11 mM glucose. As we found from static incubation studies (Fig 3.2), PWD islets had augmented insulin secretion at 11 mM glucose concentration compared to B6 islets. If PWD islets were secreting a large amount of insulin continuously overnight, then their insulin storage may be depleted relative to B6 islets. The purpose of overnight incubation was to allow islets to recover from harsh processes of islet isolation (10, 16), and the use of lower (e.g. 5.5 mM) glucose in the incubation media is not an optimal environment for islets to recover (15). Thus, the low insulin content in PWD islets may be due to the glucose concentration of the incubation media. This explanation could be examined by isolating islets then their insulin content measured immediately to see whether a difference exists between B6 and PWD mice. We suspect that the technical explanation is more likely because of
our later findings regarding pancreatic insulin area and insulin content. B6 and PWD pancreata were found to contain similar sized islet distributions, and they had similar percentages of pancreatic tissue covered with insulin suggesting a similar number of islets. In addition, the studies of the insulin content per amount of pancreas was similar between the two strains. As this was contained in a similar number of similarly sized islets, combined, this suggested that a similar amount of insulin per islet was present in vivo. Further work will be required to distinguish these possibilities.

PWD mice have hyperinsulinemia but normal glucose levels (73). The high insulin levels are consistent with the increased insulin secretion we measured, and we suspect that PWD mice have increased levels of counter-regulatory hormones to compensate for their increased insulin. Glucagon is a major counter-regulatory hormone. We examined α cell mass and did not find any differences, although differences in glucagon secretion are possible. Another explanation is also possible. Our ELISA measures all forms of insulin including proinsulin, an immature nonfunctional insulin molecule. The detection of augmented insulin secreted by PWD mice relative to B6 mice could be detecting an increased amount of proinsulin. However, we found PWD mice tolerated glucose better than B6 mice (73) suggesting that more functional insulin is secreted. Future studies should examine whether some of the insulin secreted by PWD mice is partly or fully processed.

Our results showed that insulin secretion was increased in islets from PWD mice which we suspect results in the hyperinsulinemia phenotype of PWD mice, however it was also possible that alterations in the overall morphology of the islets, the β cell mass, and the total amount of insulin present in the pancreas could contribute (83, 134). Thus we assessed these parameters in PWD and B6 mice. In terms of morphology, B6 and PWD islets were structurally
similar in terms of the overall appearance and size distributions. They resembled normal mouse islets with insulin-staining cells in the center of the islet and glucagon-staining cells surrounding. The β cell mass in PWD mice were reduced relative to B6 mice. The β cell mass accounts for both the pancreas weight and the percentage of tissue area stained with insulin. Although β cell mass was higher in B6 than in PWD mice at both four and twenty weeks of age, the difference in β cell mass was due to smaller pancreas in PWD mice. Proportionally the area occupied by β cells were similar in both strains. Thus, as there was no evidence of increased β cell mass in PWD mice we concluded that the difference between the two strains was likely due to their increased secretion.

We acknowledge a caveat in our measurement of β cell mass. Islets are unevenly distributed in the pancreas, and the areas used for staining may randomly have had more or less islets than the average distribution when only 2 to 3 sections of the pancreas were measured. Although the staining area of the entire pancreas was not measured, the preparation processes before staining the pancreatic tissues represented areas across the pancreas, and we measured multiple sections at least 300 um apart. While the limited number of sections may have increased the variability of the measurements, it was the same for all groups. Thus we believe it is unlikely that if the entire pancreas had been measured we would have specifically found an increase in the staining areas of PWD mice that would alter our conclusions.

In summary, these studies have shown that PWD mice have increased insulin secretion that likely accounts for the increased insulin levels found in vivo. Their secretion was increased specifically in response to nutrients. Our data suggest the difference is likely in the ATP generation promoting the closure of K_{ATP} (between α-KG and K_{ATP}) and unrelated to amplifying signals. Thus these studies suggest PWD mice are a valuable model to study. From discovery of
the genetic variation that cause their phenotypes, medications could possibly be created to target the altered factors, as these factors promote specifically nutrient-stimulated insulin secretion instead of increasing the risk of hypoglycemia by chronically increasing insulin levels that is a problem with some current diabetes drugs (102, 131). Future studies should be performed to identify exactly which factors are altered in the insulin secretion pathway of PWD mice. This could include the examination of the electron transport chain through identifying the ATP production level between the two mouse strains. Microarrays could also be used to identify factors creating a difference between the two strains by discovering which genes are expressed differently in islets between the two strains.
Chapter 4: Mechanisms contributing to low fasting insulin in WSB mice

4.1 Results

4.1.1 Islet architecture, size, beta cell mass and alpha cell mass

Our previous studies have shown that WSB mice have low fasting plasma insulin levels (73). To determine whether WSB mice have a defect in islet architecture or reduced β-cell mass underlying their low plasma insulin levels, we performed morphometric analyses of pancreatic sections from chow and high fat-fed WSB and B6 mice at twenty weeks of age. The islet architecture appeared normal in WSB mice (Fig. 4.1A). Their islets resembled the B6 islets and consisted an inner core of insulin staining cells surrounded by glucagon staining cells. Thus, although WSB mice had low plasma insulin levels, these data suggested that islets from WSB mice had no obvious structural defects.

To determine if the size and amount of islets were lower in WSB mice, we examined islet sizes and β-cell mass. WSB mice had more smaller islets than B6 mice on a chow diet (Fig. 4.1B). When these mice were fed with a HFD, B6 had an increase in larger sized islets while no significant effects of high fat feeding were observed in WSB mice. Thus, the mean islet size in high fat-fed WSB mice was significantly reduced compared to B6 mice (Fig. 4.1C). No significant differences in insulin staining areas as a percentage of pancreatic area examined were observed (Fig 4.1D). Similar to PWD mice, we also found that the pancreas weight was significantly lower in WSB mice than in B6 mice (Fig. 4.1E). Thus, total β-cell mass was significantly lower in WSB mice than in B6 mice fed chow, with similar trends in mice fed the HFD (Fig. 4.1F). Because previous insulin tolerance tests suggested WSB mice may have a more robust counter-regulatory response (73), we also examined the glucagon staining area in the
pancreas but found no difference between the strains on either diet (Fig 4.1G). However, given the reduced size of the pancreas in WSB mice, total α-cell mass was lower in chow fed WSB compared to B6 mice (p = 0.045) and tended to be lower in HFD fed mice (Fig 4.1H). In conclusion, these data suggest that reduced β cell mass may contribute to the low plasma insulin levels observed in WSB mice relative to B6 mice at 20 weeks of age.
Figure 4.1. Islet architecture and morphometry at twenty weeks of age in WSB and B6 mice. 

A, Representative islets from B6 and WSB mice showing islet morphology. Blue DNA (DAPI), red insulin, green glucagon. 

B, Islet size distribution. Black B6, white WSB, solid colour chow diet, striped HFD. 

C, Mean islet size. 

D, Percentage of pancreatic tissue (DAPI-staining) area stained positively for insulin. 

E, Pancreas weight. 

F, Total β cell mass. 

G, Percentage of pancreatic tissue (DAPI-staining) area stained positively for glucagon. 

H, Total α cell mass. 

*p<0.05,**p<0.01,***p<0.001 B6 vs. WSB mice consuming the same diet. *p<0.05,**p<0.001 Chow vs. HFD fed of the same strain. (n = 5 per strain per diet).
Insulin resistance is induced by feeding on a HFD and can also be affected by age (117, 147). Once the body becomes resistant to insulin, the β cells proliferate in order to compensate for the increased need for insulin (38, 115). At twenty weeks of age, B6 mice had developed insulin resistance (73). As opposed to B6 mice, WSB cleared glucose normally at this age with no sign of insulin resistance even when fed with the HFD. Both chow and HFD fed WSB mice were more insulin sensitive than B6 mice at this age (73). Thus, it was not surprising to see that B6 mice had expanded their islet sizes and β cell mass. This may have explained why WSB had a lower β cell mass compared to the insulin resistant B6 mice.

In order to determine the WSB phenotype without the potential effect of insulin resistance and to determine whether WSB mice had an inherent defect in β cell mass, we re-examined these parameters at four weeks of age. As at twenty weeks of age, islet architecture appeared normal in WSB mice (Fig. 4.2A). We examined islet areas in the sections to determine whether WSB mice have smaller islets. In contrast to what was observed at 20 weeks of age, chow-fed WSB mice had a lower percentage of small islets (p< 0.01) and tended to have a higher percentage of larger islets compared to chow-fed B6 mice (Fig 4.2B). This resulted in a significantly larger mean islet size in WSB mice compared to B6 mice (Fig 4.2C). Similar patterns were observed in mice fed the HFD from weaning at 3 weeks of age (Fig 4.2B), however no significant difference in mean islet size was observed (Fig 4.2C). This suggested that there were no obvious developmental defects, as WSB mice were able to produce normal sized islets. Although the mean islet sizes were increased in chow-fed WSB mice, to determine if total β-cell mass in WSB mice was reduced such as if the number of islets were reduced, we examined the percentage of insulin staining area in the pancreas. We found WSB mice tended to have an increased staining area compared to B6 (Fig. 4.2D), consistent with their larger islets. Unlike at
20 weeks of age, at 4 weeks of age, pancreatic weights were not significantly different between the strains (Fig. 4.2E). Therefore, total β-cell mass in young WSB mice was not significantly different than B6 mice at this age (Fig. 4.2F). We also examined the glucagon staining area and found no significant difference, although consistent with their tendency for increased islet sizes it was slightly higher in chow-fed WSB mice (Fig 4.2G, H).
Figure 4.2. Islet architecture and morphometry at four weeks of age in WSB and B6 mice. A, Representative islets from B6 and WSB mice showing islet morphology. Blue DNA (DAPI), red insulin, green glucagon. B, Islet size distribution. Black B6, white WSB, solid colour chow diet, striped HFD. C, Mean islet sizes. D, Percentage of pancreatic tissue (DAPI-staining) area stained positively for insulin. E, Pancreas weights. F, β cell mass. G, Percentage of pancreatic tissue (DAPI-staining) area stained positively for glucagon. H, α cell mass. **p<0.01 B6 vs. WSB mice consuming the same diet. *p<0.05 Chow vs. HFD fed mice of the same strain. (n = 5 mice per strain per diet).
As β-cell mass is determined by staining area and does not account for the insulin content of the stained area, we also examined total pancreatic insulin content in young mice (7 weeks of age) to determine whether islets from WSB mice contained less insulin. Total insulin per gram of pancreas was not different between the strains (Fig. 4.3A). At this age, like at 2 weeks, we found a much larger pancreas in the B6 mice than in WSB mice (Fig 4.3B). This increased pancreas size in the B6 mice augmented significantly the total amount of insulin content within the pancreas in B6 mice compared to WSB mice on the HFD, with a similar trend on the chow diet (Fig 4.3C).

At four weeks of age (Fig 4.2E), we found that WSB mice had a similar pancreas weight as B6 mice. At seven weeks of age here, we determined that WSB mice maintained a similar pancreas weight as at four weeks, whereas the pancreas size in B6 mice doubled (Fig 4.3B). The pancreas size in WSB mice was similar to that observed even at twenty weeks (Fig 4.1E), while by seven weeks the B6 pancreas had reached a size only slightly smaller than that observed at twenty weeks of age (Fig 4.3B). This suggested that between four and seven weeks of age or early on in life, WSB began to have post-natal growth defect, resulting in a smaller pancreas and lower β cell mass and insulin content that may contribute to their low fasting insulin throughout their life.
Figure 4. Whole pancreas insulin content in WSB and B6 mice at seven weeks of age. Black B6, white WSB, solid colour chow diet, striped HFD. A, Pancreatic insulin content per gram of pancreas. B, Pancreas weight. C, Total pancreatic insulin content.

*p<0.05, ***p<0.001 B6 vs. WSB mice consuming the same diet. ++p<0.01 Chow vs. HFD fed of the same strain. (n = 5-6 WSB per diet, n = 10 B6 per diet)
4.1.2 Insulin secretion

As WSB mice also secreted low insulin in response to a glucose challenge in vivo (73), the secretory function of the WSB islets was investigated by perifusion to determine whether it is also impaired. These perifusion studies were performed similarly as for PWD mice as described above with islets from ~6 week-old B6 and WSB mice. Surprisingly, insulin levels in the perfusates from WSB islets were approximately 3 times higher than those from B6 mice when stimulated with 20 mM high glucose (Fig. 4.4A). The tripling of the insulin levels observed in the perfusate resulted in approximately 7 times more total insulin released from WSB islets compared to B6 islets (Fig. 4.4B). The pattern of first and second phase insulin secretion was observed in both strains, demonstrating functionally intact secretion in WSB mice. The amount of insulin secretion at basal glucose concentrations (3 mM) was similar between WSB and B6 mice, with if anything a trend to lower insulin secretion from WSB islets. This further showed that the islets were functioning properly, and were not leaky or releasing their content in the absence of stimulation.

When insulin secretion was triggered with a mixture of 30 mM potassium chloride (in 3 mM glucose), insulin levels in the perfusate from WSB islets was roughly doubled relative to B6 islets (Fig. 4.4A). As a result, islets from WSB mice secreted nearly 3 times more insulin in response to potassium compared to B6 islets (Fig. 4.4C). This suggested that although secretion in response to glucose stimulation in vivo was reduced (73), WSB β-cells were capable of secreting a high level of insulin.
Figure 4.4. Insulin secretion in islets from WSB mice compared to B6 mice. Perifusions were performed in ~6 week old mice. A, Insulin secretion response of B6 and WSB islets in 3 mM basal glucose, 20 mM glucose, and 30 mM KCl for the times indicated. Thin line B6 islets, Thick line WSB islets. B, Black B6, white WSB Area between insulin secretion curve and average basal glucose insulin response when incubated with 20 mM glucose. C, Area between insulin secretion curve and average basal glucose insulin response when incubated with mixture of basal glucose and 30 mM potassium chloride. **p<0.01, ***p<0.001 B6 vs WSB islets triggered by same secretagogue. (n = 10 per strain).
4.1.3 Islet vascularization

The preceding data have shown that there is a difference in insulin secretion in vivo versus when the islets are isolated and examined in vitro. One factor that differs between these two situations is islet vascularization (39). Vascular density within islets has been shown to affect secretion in vivo (108). To determine whether vascularization is compromised in WSB mice, we examined CD31 staining as a marker of endothelial cells in the islet (108). We were unable to show a difference in the amount of islet vascularization (CD31 staining area within islets) between WSB and B6 islets at four weeks of age (Fig. 4.5). This suggested that vascular density of the islets was likely not the factor affecting insulin secretion from WSB islets in vivo.

![Figure 4.5. Islet vascularization at 4 weeks of age.](image)

Percentage of islet (area selected based on the insulin staining) area stained positively for CD31. (n = 5 mice per strain with 2 sections per mouse with approximately 100 islets examined per strain)
4.2 Discussion

In previous studies, we found WSB mice maintained low fasting plasma insulin levels with age and high fat feeding and secreted low amounts of insulin relative to B6 mice in response to a glucose challenge in vivo (73). In B6 mice, the pancreas doubled in weight comparing four weeks to twenty weeks of age (p <0.001). In contrast, the size of the pancreas in WSB mice, although not different to B6 mice at 4 weeks of age, remained relatively constant over time. This difference in the rate of increase in pancreas weight between B6 and WSB not only occurred in the B6 mice that became insulin resistant after feeding with a high fat diet, but it occurred in chow fed mice as well. The findings from chow fed B6 mice reflected the regular β cell development occurring in normal rodents. Furthermore, at 7 weeks of age, both strains did not show a difference in insulin sensitivity, but B6 mice had a much bigger pancreas than WSB mice which did not grow any bigger after 7 weeks. Overall, WSB mice were much smaller and differed from B6 mice in both pancreas and body size during the period of 4 to 7 weeks. Thus, there could be a difference in post-natal pancreatic growth between the two strains, rather than changes in pancreatic size to compensate for insulin resistance. The reduced pancreas size in WSB mice was associated with a reduced β cell mass and pancreatic insulin content. This may contribute to the low insulin levels of adult WSB mice. Defects in postnatal pancreatic growth can result in hyperglycemia which then is associated with diabetes (58).

Prior to T2D development, the body becomes more resistant to insulin, requiring increased insulin production to maintain glucose homeostasis (58). This is achieved by increasing insulin secretion and/or increasing β-cell mass in order to compensate for the amount of insulin needed (4). Comparing the islet size distributions, it can be seen that B6 islets became larger at twenty weeks of age, especially after high fat feeding. In B6 mice, the increased
pancreatic and islet sizes led to approximately tripling of their β-cell mass at twenty weeks of age versus four weeks of age for both diets. Some islets from WSB mice also increased in size, as both strains had islets with areas >10000 µm² by 20 weeks of age that were not detected at 4 weeks of age. However, in WSB mice, while some larger islets were present, there was a higher fraction of small islets, that resulted in a reduction in mean islet size at 20 weeks versus 4 weeks of age. For β-cell mass in WSB mice, no increase was seen between 4 and 20 weeks of age for both diets, suggesting that aging and high fat diets themselves were insufficient in promoting an increase in β-cell mass in WSB mice. It is unclear whether the consistent low β cell mass and insulin in WSB mice prevented them from becoming insulin resistant (80), or conversely if the lack of insulin resistance prevented the need for WSB mice to increase β cell mass and insulin (84).

Our prior studies showed that WSB mice have an increased counter regulatory response to low glucose (73). Glucagon produced by α cells is one of the main counter regulatory hormones (73). Therefore, we also examined α cells. In addition, examining α cells permits us to determine the balance between insulin and glucagon function in maintaining the normal glucose levels despite low insulin in WSB mice. The percentage of α cell area in B6 mice decreased, but their total α cell mass remained the same from 4 to 20 weeks of age. This suggested that the total number of α cells was similar and that the pancreas and the amount of β cells but not α cells increased between 4 to 20 weeks in B6 mice. In WSB mice, the percentage of α cell area and α cell mass decreased between 4 to 20 weeks and that the pancreas size did not change. This suggested a loss of α cells. During this time, WSB mice did become more insulin sensitive. This is an interesting finding because the high insulin sensitivity in WSB mice may have prevented
the need for much glucagon, resulting in a decrease in α cells through an unknown mechanism. Future studies will be required to confirm this finding.

Although the lower β-cell mass in WSB mice likely contributes to their lower insulin secretion in vivo, we were surprised to find a robust response of their islets in vitro, when previously we consistently found little to no response to a glucose challenge in vivo (73) in multiple cohorts across multiple time points. In contrast, the perifusion results showed that insulin secretion was substantially higher from WSB islets in response to both glucose and potassium compared to B6 islets. Unlike PWD mice, the pattern for first and second phase of the insulin secretion were consistent between B6 and WSB mice. The perifusion studies were performed on young mice with hand-picked islets matched for size between WSB and B6 mice. Thus, this finding is not likely simply due to the larger average islets in WSB mice relative to B6 mice at this age. Since WSB mice can produce robust insulin secretion, this suggested that their low insulin response in vivo may result from their increased insulin sensitivity and the reduced need for insulin (101).

The pancreas, and particularly islets, are densely vascularized (106). Although the pancreas consists of approximately one to two percent of islets (111), ten percent of pancreatic blood flows into these islets (108). Although the degree of vascularization was similar, we cannot exclude differences in vessel structure between WSB and B6 mice. Islet endothelial cells lie in the inner part of the blood vessels (39), which are covered with pericytes. The islets receive nutrient and hormonal signals for insulin secretion from the blood travelling to pancreas through crossing the endothelial cells (108). This means that any blockage of these signals from the endothelial cells such as increased pericyte density or reduced fenestrations/pores in the endothelial cells could affect the passage of molecules, such as nutrient and hormonal signals, to
the β-cells and thus the amount of insulin secreted. Other potential reasons affecting insulin secretion in WSB mice *in vivo* could include altered vascular structure (101), changes in islet innervation (101), or perhaps a circulating factor that reduced insulin secretion in WSB mice. Future studies will be required to determine the mechanism underlying this difference between the insulin secretion observed *in vitro* and *in vivo* in WSB mice.

In summary, these studies suggested that WSB mice have reduced post-natal pancreatic growth that led to reduced β cell mass in adults and may have contributed to the maintenance of their low insulin levels. This makes WSB a good model to discover novel mechanisms postnatal pancreatic growth (101). Future experiments should aim to determine the signals affecting post-natal pancreatic growth in WSB mice. Additionally, we unexpectedly found WSB islets to have increased secretion *in vitro*. The physiological mechanisms for the increased insulin levels are regulated by genetic factors, thus WSB mice are good model for the discovery of novel genetic factors affecting insulin secretion (5, 44, 50, 62), providing new insights into the processes that could lead to novel treatment in T2D.
Chapter 5: Discussion and conclusion

5.1 Discussion and Conclusion

PWD and WSB mouse strains are relatively novel in the research field. They are not well studied yet as they have not been around long enough, so we do not fully understand them. In fact, they are rarely studied in diabetes research although they do express diabetes related characteristics.

The PWD mouse strain is a primary hyperinsulinemia mouse model with a late onset of insulin resistance and obesity induced with the HFD (73). Examination of PWD pancreatic islets in this thesis suggests that there may be altered factors in the insulin secretion pathway augmenting insulin secretion in PWD mice relative to B6 mice. These altered factors are likely present prior to the depolarization step in the insulin secretion pathway, such as in the generation of ATP, although a factor having a more minor effect may also exist downstream of depolarization.

There are many inbred mouse models used in diabetes research exhibiting hyperinsulinemia. However, this is usually accompanied with insulin resistance. For example, New Zealand obese (NZO) mouse strain has severe hyperinsulinemia, and this is associated with the reduction of insulin stimulated glucose uptake ability by the skeletal muscle and adipose tissue (19). Another example is the KK mice, they developed hyperinsulinemia after becoming glucose intolerant. KK mice also develop obesity and hyperleptinemia (19). Unlike NZO and KK mice, the hyperinsulinemia of PWD mice is not accompanied with insulin resistant or obesity until later in life when fed with HFD (73). There is a common dogma that insulin resistant occurs prior to hyperinsulinemia, but this may not be the case in PWD mice. DBA/2 mouse strain is
commonly used in diabetes research (19). After inducing insulin resistance through heterozygous insulin receptor and insulin receptor substrate-1 alleles, they have similar traits as PWD. DBA/2 mice are more glucose tolerant than B6 yet higher insulin secretion (19). They differ from PWD mice as their increased insulin secretion is associated with increased β cell mass (19). This makes DBA/2 mice more prone to β cell failure that could possibly lead to diabetes (19).

Comparatively, there was no significant difference found in our studies between B6 and PWD mice for β cell mass. PWD mouse strain is unique because its hyperinsulinemia is not shown to be accompanied with other diabetes related traits. Future genetic studies using PWD mice are likely to identify factors in augmenting insulin secretion without increasing diabetes related characteristics.

To address the altered factor upstream of depolarization, future studies could examine the ATP to ADP ratio produced by the mitochondria of PWD islets and B6 islets. For example, Perceval is a reporter protein composed of green fluorescent protein and a regulatory protein GLNK1 which is able to detect changes in the ATP to ADP ratio in a living cell (8). The regulatory protein can bind to both ATP and ADP molecules, but it differs in the level of its fluorescence after the binding (8). As ATP and ADP molecules compete to bind to GLNK1, their binding ratio will reflect the concentration ratio present in the cell (8). We hypothesize that PWD islets will have augmented ATP to ADP ratio relative to B6 islets.

Additionally, future studies could examine possible altered factor downstream of depolarization in the insulin secretion pathway in PWD and B6 islets. From our perifusion studies, PWD islets secreted more insulin than B6 islets. Moreover, B6 islets exhibited the expected biphasic insulin secretion, yet PWD islets had minimal difference between the two phases. A similar pattern was seen in a previous study after the interruption of actin filament
Actin filament prevents the translocation of insulin containing granules to the plasma membrane unless stimulated by a nutrient (132). Disrupting the function of actin filament through the use of Latrunculin B induced an augmentation in both phases of insulin secretion and minimal difference was shown between the two phases (132). Thus, we could examine the actin filament in PWD islets compared to B6 islets to verify a possible alteration resulting in the PWD mice insulin phenotype. We hypothesize that PWD mice will have altered actin filament allowing the efficiency of insulin secretion. Factors within the insulin secretion pathway may lead us to the mechanism for the hyperinsulinemia of PWD mice.

WSB mice have low fasting insulin and increased insulin sensitivity relative to B6 mice (73). WSB mice are also resistant to diet-induced obesity (73). Their low fasting insulin levels could be protecting them from developing both obesity and insulin resistance (80). From our studies, we found that WSB mice may have reduced post-natal pancreatic growth combined with factors restricting insulin secretion in vivo.

The insulin phenotypes of WSB mice are similar to E2f1 knockout mice with B6 background (40). These knock out mice exhibited reduced postnatal pancreatic growth resulting in a reduced pancreas size (40). Their islets, possibly due to postnatal growth defect, secreted low levels of insulin but the mice did not experience glucose intolerance because they had augmented insulin sensitivity (40). Thus, future studies should examine the E2f1 gene as this gene may be responsible for WSB mice phenotypes. For example, examining the gene expression level of E2f1 between WSB mice and B6 mice through the use of real-time polymerase chain reaction. We hypothesize that WSB mice will have a lower expression level of E2f1 relative to B6 mice in order to exhibit the similar phenotypes of the E2f1 knockout mice.
Additional future studies specifically addressing the difference in insulin secretion in WSB mice between *in vivo* and *in vitro* studies could include examining the blood vessel structure, nerves around the pancreatic islets, or possible signal and hormone affecting the transportation or the degradation of insulin molecules. For example, we could examine the concentration of epinephrine, norepinephrine, or α-adrenoceptor agonist in the pancreatic islets. These agents inhibit insulin secretion from pancreatic islets during a sympathetic nervous system stimulation (60). We hypothesize that WSB islets will have augmented level of these agents compared to B6 islets.

Wild-derived inbred strains will help to resolve the shortcoming of the limited genetic variability in the classical inbred mouse strains. Here, we examined factors contributing to type 2 diabetes risk (insulin secretion and β-cell mass) in two wild-derived inbred strains. We found that PWD had altered factors within their insulin secretion pathway leading to increased insulin secretion relative to B6. For WSB mice, we found that the low insulin secretion may result from reduced post-natal pancreatic growth combined with factors restricting insulin secretion *in vivo*. Surprisingly, we discovered that WSB mice have highly increased insulin secretion *in vitro*. Identifying the physiological mechanisms of PWD and WSB mice for T2D risk factors indicate that their genetic variation will give us clues regarding factors to enhance insulin secretion and to protect from T2D respectively. Future identification of the genes responsible for these physiological mechanisms will allow us to better understand the regulation of T2D and to develop novel medications and treatments for T2D. Therefore, PWD and WSB are valuable mouse models opening a new world of undiscovered T2D knowledge.
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