Mechanistic Role of ARNT/HIF-1β in the Regulation of Glucose-Stimulated Insulin Secretion

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

Loss of glucose-stimulated insulin secretion (GSIS) from the pancreatic beta-cells is one of the earliest detectable defects in the pathogenesis of type 2 diabetes. However, despite its relevance, the mechanisms that govern GSIS are still not completely understood. ARNT/HIF-1β is a member of the bHLH-PAS family of transcription factors, with a prominent role in the transcriptional regulation of enzymes required for the metabolism of xenobiotics as well as regulation of genes that are critical for cellular responses to hypoxia. Recent research has uncovered a previously unknown function for ARNT/HIF-1β in the pancreatic beta-cells, where the gene was found to be 90% down-regulated in human type 2 diabetic islets and loss of ARNT/HIF-1β protein leads to defective GSIS in pancreatic beta-cells of mice. The main focus of this thesis was to understand the mechanisms by which ARNT/HIF-1β maintains normal GSIS from pancreatic beta-cells and understand how loss of ARNT/HIF-1β leads to beta-cell dysfunction and type 2 diabetes in mice. ARNT/HIF-1β was found to positively regulate GSIS in both INS-1 derived 832/13 cell line and mice islets. In the 832/13 cells, loss of ARNT/HIF-1β leads to a reduction in glycolysis without affecting the glucose oxidation and the ATP/ADP ratio suggesting that the regulation of GSIS takes place in a manner that is independent of the K_{ATP} channels. In order to further assess the mechanism of lowered GSIS in the absence of ARNT/HIF-1β in the 832/13 cells, a metabolite profiling was performed which revealed a significant reduction in the metabolite levels of glycolysis and the TCA cycle intermediates and glucose-induced fatty acid production, suggesting the involvement of ARNT/HIF-1β in regulating glucose-stimulated anaplerosis, which is believed to play a key role in the regulation of GSIS from the pancreatic beta-cells. The changes in metabolite levels in the absence of ARNT/HIF-1β were associated with corresponding changes in the gene expression pattern of key enzymes regulating glycolysis, the TCA cycle and fatty acid synthesis in beta-cells. In an attempt to understand how loss of ARNT/HIF-1β leads to beta-cell dysfunction and type 2 diabetes in mice, a pancreatic beta-cell specific ARNT/HIF-1β knock out mouse (β-ARNT KO) was generated using the Cre-loxP technology. Functional characterization of islets from both male and female β-ARNT KO mice revealed a significant impairment in GSIS, which was

attributed due to a small, but significant reduction in rise in intracellular calcium upon glucose stimulation. Further analysis revealed reduced secretory response to glucose in the presence of KCl and diazoxide indicating a defect in the amplifying pathway of GSIS in β-ARNT KO islets. Expression of pyruvate carboxylase (PC) was significantly reduced in β-ARNT KO islets suggesting possible impairments in anaplerosis and consistent with this, defect in GSIS in β-ARNT KO islets could be almost completely rescued by treatment with membrane permeable TCA intermediates. Surprisingly, both male and female β-ARNT KO mice have normal glucose homeostasis. In an attempt to assess how β-ARNT KO mice maintained normal blood glucose levels, indirect calorimetry was used to understand changes in whole-body energy expenditure. This investigation revealed that β-ARNT KO mice exhibited a small but significant increase in respiratory exchange ratio (RER), suggesting a preference in utilizing carbohydrates as a fuel source, possibly leading to improved glucose uptake from the blood stream. Response to exogenous insulin was completely normal in β-ARNT KO mice suggesting intact functioning of the skeletal muscles. To conclude, based on our *in vitro* data, we believe that ARNT/HIF-1β plays an indispensable role in maintaining normal beta-cell secretory function, however, results from β-ARNT KO mice indicates that these mice are protected from the adverse effects of hyperglycemia. Although loss of ARNT/HIF-1β alone is not sufficient for the genesis of type 2 diabetes, it creates a perfect storm in the pancreatic beta-cells that may eventually lead to an imbalance in the whole body glucose homeostasis. Our study provides significant information to the scientific community that engages in assessing the pharmacological potential of gene targets for the treatment of type 2 diabetes.

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DEDICATION

For their constant encouragement, mentoring, guidance and support throughout my life and throughout my PhD, I dedicate my thesis to my parents Dr. P.P.Pillai and Dr. Lakshmy Devi Pillai, my incredibly wonderful and supportive husband Hari Menon, my son Rishabh Menon and my two sisters Dr. Renuka Pillai and Rohini Pillai. You have ALWAYS believed in me and for that, I am forever indebted to you.

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Introduction

1.1 Rationale

Diabetes mellitus, a disease originally described as a condition producing excessive thirst (polydipsia), excessive hunger (polyphagia) and excessive urination (polyuria), has been affecting human lives for thousands of years and was one of the first diseases recorded in the ancient literature, dating as far back as 1500 BC in the Egyptian manuscripts (1,2). Around the same time, physicians in India reported that urine from people presented with the above clinical symptoms attracted ants and flies and named the condition as "madhumeha" or "honey urine". Although diabetes mellitus has been recognized as an illness for centuries and physicians in different parts of the world have been treating the disease with various efficacies, the prognosis for the disease in those days were shockingly grim and the disease was generally considered as a death sentence. In 1889, Joseph Von Mering, a German physician and Oskar Minkowski, a Russian scientist, discovered the role of pancreas in diabetes, when they observed that pancreatectomized dogs displayed all clinical symptoms of diabetes mellitus and died shortly after the pancreatectomy. However, it was not until early 1900s that an effective and life saving treatment for diabetes mellitus was discovered with the advent of the hormone insulin from the pancreas by Canadians Dr. Frederick Banting and Dr. Charles Best. Ever since that historic moment in 1922, scientists across the globe have been working relentlessly to understand the causes and the pathophysiological abnormalities associated with diabetes mellitus, generating a wealth of knowledge in the field. This new found knowledge about the disease has led to the discovery of many new treatments options for diabetes mellitus in the 20^{th} century and has improved the quality of life of thousands of diabetics, who otherwise had a very grim prognosis.

Despite the commendable advancements made in diabetes research in the last century and a half, the complex and multifaceted nature of the disease has made it nearly impossible for scientists to understand the exact nature of the causes and consequences of the disease. Although scientific discoveries have led to effective strategies for the prevention of diabetes,

pathway to cure still remains an elusive dream. The clinical presentation of diabetes has also tremendously changed from a century ago to now, from severe insulin deficiency being one of the critical symptoms, to the current clinical presentation, where insulin resistance associated with impaired insulin secretion plagues the vast majority of diabetics. The fact that these clinical symptoms are a consequence of over-eating and obesity has made diabetes mellitus a global epidemic and one of the leading causes of reduced quality of life and life expectancy in the 21st century.

In the past century or so, dramatic advancements have been made in the understanding of metabolic regulation of insulin secretion and maintenance of normal glucose homeostasis in humans. The fact that impaired insulin secretion is one of the most clinically relevant abnormalities presented in the current generation of diabetics and the ballooning population of diabetics across the globe, makes the research in unraveling the mechanisms that regulate insulin secretion from pancreatic beta-cells more relevant than ever before. Although, the basic mechanisms that govern insulin release have been well studied and some of the key metabolic pathways involved in the secretion of insulin have been discovered, the exact nature of these pathways and the identity of the metabolic signals that control insulin secretion is still not completely understood. Therefore, a complete understanding of these intricate processes is critical in finding better treatment options for the prevention or cure of diabetes. My thesis is an attempt to broaden our understanding of the metabolic control of insulin release from the pancreatic beta-cells and how it helps sustain normal glucose homeostasis in mice. Insulin secretion can be regulated through different ways in our body and it is a well-known fact that dietary carbohydrates, specifically glucose, are one of the major regulators of insulin. It is therefore necessary that we gain a better understanding of the mechanisms that govern glucose-stimulated insulin secretion (GSIS) and the key regulators that play a critical role in governing these mechanisms. Specifically, my thesis will examine the role of transcription factor, aryl hydrocarbon receptor nuclear translocator (ARNT)/hypoxia inducible factor-1β (HIF-1β) in the regulation of GSIS and maintenance of normal glucose homeostasis in mice. However, before I delve into the details of my research

topic, the following parts of this chapter will focus on providing the required background information on research in diabetes.

1.2 Pancreatic Islets of Langerhans and Insulin

The pancreas is a soft, elongated, lobular organ that is located on the posterior abdominal wall, close to the stomach (3). It has both an exocrine part and an endocrine part. The exocrine part of the pancreas, known as the acinar tissue, is formed of zymogenic cells and secretes digestive enzymes. The endocrine part of the pancreas known as the pancreatic islets or the islets of Langerhans, synthesizes and secretes hormones. Human pancreas has roughly one million pancreatic islets (4). Each islet is a heterogeneous population of 5 kinds of cells namely, alpha-cells, beta-cells, delta-cells, epsilon-cells and pancreatic polypeptide cells (PP). Alpha-cells secrete the hormone glucagon, beta-cells secrete insulin, delta-cells secrete somatostatin, epsilon-cells secrete ghrelin and PP cells secrete pancreatic polypeptide. Among the different kinds of cells, beta-cells are the most prominent cells as they occupy 60-70% of the islet volume. Interestingly, different types of cells in the islets are distributed in an orderly fashion, although the pattern of distribution is different in rodents and humans (5,6). In humans, beta-cells are scattered across the islet, whereas in rodents, beta-cells form the core of the islet, surrounded by a ring of alpha, delta and PP cells. Human pancreas is abundantly innervated and rich in blood supply and the islets were thought to be innervated by both sympathetic and parasympathetic nerves. However, recent studies demonstrate that human islets are scarcely innervated and the innervation pattern is quite different from that observed in mouse islets, which were found to be abundantly innervated (7). Compared to mouse islets, the axonal density and axon contact points are relatively sparse in human islets. In human islets, a few parasympathetic cholinergic axons penetrate the islet and the invading sympathetic fibers preferentially innervate smooth muscle cells of blood vessels located within the islet. Thus, contrary to the age-old belief that the autonomic nervous system regulates hormone secretion directly from the endocrine pancreas, recent discoveries by Rodriguez et al (7) suggest that sympathetic nerves may regulate hormone secretion in human islets by controlling the local blood flow within the endocrine pancreas. These unique

differences between the human and rodent islets make one wonder about the functional implications it can have on the islet cell function.

Islets of Langerhans were first described by Paul Langerhans in 1869 (8), however, the hormone insulin was not discovered until 1921 when Nobel laureate Dr. Fredrick Banting along with Dr. Charles Best from the University of Toronto established that pancreatic islets secreted a hormone, which when delivered systemically, was capable of curbing hyperglycemia in diabetic dogs and can be used for the treatment of type 1 diabetes (9-12) Insulin, often called the "miracle drug" of the 20th century, soon became the elixir of life for children and adults dying from diabetes. Decades after the initial discovery, the amino acid structure of insulin was characterized by Frederick Sanger in 1951, for which he was awarded the Nobel Prize in Chemistry in 1958 (13,14). Compared to other macromolecules, insulin is a small protein made up of 51 amino acids and consists of two polypeptide chains, named subunit A and B held together by disulphide bonds. Insulin serves several important functions in vertebrates (15). It is the master regulator of glucose and lipid homeostasis and is secreted primarily in response to a post-prandial hike in blood glucose concentration. In addition to glucose, other fuel molecules such as amino acids, hormones and some neural stimuli can also stimulate the secretion of insulin from the beta-cells of pancreas. The physiological role of insulin in maintaining whole body glucose homeostasis is associated with its ability to promote the uptake and storage of glucose by the peripheral tissues namely, the skeletal muscles, the liver and the adipose tissue. Insulin also inhibits the production of glucose through glycogenolysis from the liver in its attempt to maintain circulating glucose levels in the normal physiological range. In addition to its role in maintaining glucose homeostasis, insulin also serves as an anabolic hormone by promoting the uptake of amino acids, contributing to DNA replication and protein synthesis.

1.3 Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic syndrome characterized by chronic hyperglycemia resulting from impaired insulin secretion from the pancreatic beta-cells or defective insulin action on the peripheral tissues such as the skeletal muscles or a combination of these

abnormalities (16). Chronic hyperglycemia can have serious impact on different organs of our body leading to both microvascular and macrovascular complications. Some of the devastating side effects of prolonged hyperglycemia are hypertension, high cholesterol and high triglyceride levels leading to cardiovascular diseases and stroke, diabetic retinopathy leading to complete loss of vision, diabetic nephropathy leading to kidney failure, peripheral neuropathy leading to foot ulcers, amputations and sexual dysfunction. With an estimated 347 million people afflicted world wide, the World Health Organization (WHO) predicts that complications from diabetes will be the 7th leading cause of death by the year 2030 compared to its. Aside from the obvious personal cost to people with diabetes, which includes reduced life span, the financial burden associated with treatment of diabetes to patients as well as governments across the globe is humungous. According to the Canadian Diabetes Association, by 2020, diabetes will cost the Canadian healthcare system an estimated $16.9 billion a year. From a clinical standpoint, DM can be classified into three main categories namely, type 1, type 2 and gestational diabetes. The pathophysiology of type 1 and type 2 diabetes are described in detail in section 1.3.1 and 1.3.2. Gestational diabetes, characterized by the classical hyperglycemia, is first diagnosed during pregnancy. Unlike the other two forms of diabetes, gestational hyperglycemia is a temporary phenomenon and most women are relieved of symptoms at the end of their pregnancy. However, women with gestational diabetes are at greater risk to develop type 2 diabetes at a later stage of their life (17).

1.3.1 Type 1 diabetes (T1D)

T1D, also known as juvenile diabetes or insulin dependent diabetes mellitus, is caused by the autoimmune destruction of the pancreatic beta-cells (18). Representing 10% of the diabetic population, T1D leads to a complete lack of insulin production, leading to chronic hyperglycemia in patients. One of the hallmark features of T1D is the infiltration of the islets by cells of the immune system, leading to inflammation and a condition known as insulitis. Markers of the autoimmune destruction of the beta-cells include autoantibodies to insulin, glutamate decarboxylase 65 (GAD65), and autoantibodies to the tyrosine phosphatases IA-2

and IA-2β (19,20). In 2008, Ludviggson et al showed that subcutaneous injection of GAD-alum, an adjuvant-formulated vaccine incorporating human recombinant GAD65, preserved insulin production for up to 30 months in type 1 diabetics, who were screened positive for GAD autoantibody (21). Although administration of GAD-alum slowed down the loss of residual beta-cells, it did not change insulin dependency for these patients. Usually, 85-90% of the type 1 diabetics will have the presence of one or more of these autoantibodies when fasting hyperglycemia is initially detected. The risk of developing T1D is strongly related to the number of autoantibody markers, where the presence of two or more autoantibodies gives a higher probability of developing the disease than the presence of a single autoantibody. In T1D, it is generally believed that the rate of beta-cell destruction is quite varied, where infants and children are observed to have a rapid rate of destruction compared to adults. Although, it is most commonly diagnosed in children, T1D can occur at any age of human life.

Despite the significant advancements made in T1D research in the last several decades, the exact mechanisms underlying the initiation and progression of beta-cell destruction are still very poorly understood (22-24). Most of our knowledge on the pathogenesis of T1D comes from studies conducted in animal models of T1D namely, non-obese diabetic (NOD) mice and the biobreeding-diabetes prone (BB-DP) rats. Even though we do not know what triggers the infiltration of islets by the immune cells, it is generally believed that beta-cell autoantigens, macrophages, dendritic cells, B lymphocytes and T lymphocytes are all involved in beta-cell autoimmune destruction. One of the major obstacles to unveiling the molecular and cellular basis of disease progression in T1D is the lack of availability of pancreatic specimens in which islets are undergoing autoimmune destruction. Currently, the only effective treatment option with guaranteed results for T1D is daily doses of insulin injections to keep the blood glucose level in a normal and healthy range. However, a method that has also gained a lot of momentum in the last decade or so is the process of islet transplantation for people suffering from T1D (25). Several islet cell transplantation clinical trials are underway across the globe. Although the process has a promising future, as with any organ transplantation procedures, the related complications such as immunogenicity,

decline in graft function and continued dependence on exogenous insulin injections for maintaining normoglycemia, all point to a procedure that requires a substantial amount of improvement before it becomes a mainstream treatment option for type 1 diabetics.

1.3.2 Type 2 diabetes (T2D)

T2D, also known as the non-insulin dependent diabetes mellitus or adult on-set diabetes, is characterized by hyperglycemia due to insulin deficiency or peripheral insulin resistance or both (26). Representing 90% of the diabetic population, T2D has now become a global epidemic, affecting millions of people world-wide. Pancreatic beta-cell dysfunction is one of the hallmark features of T2D (27). A decline in beta-cell function as determined by changes in glucose tolerance from normal to glucose intolerant and loss of glucose-stimulated insulin secretion, particularly the first phase of insulin release, are some of the earliest events in the progression of the disease (28). The failure of beta-cells to secrete a sufficient amount of insulin could be attributed to several factors, the two most prominent being, abnormalities in beta-cell glucose metabolism and a decrease in beta-cell mass (29,30). Under normal conditions, pancreatic beta-cells have an incredible capacity to increase their mass to adapt to changing insulin demands of the body such as in pregnancy or weight gain. Beta-cell replication, neogenesis and hypertrophy are the factors that contribute to increase in beta-cell mass in humans and rodents (31). In pre-diabetics or people diagnosed with T2D, beta-cells fail to increase their mass and compensate for the increased insulin demands. This leads to progressive elevation in plasma glucose levels, culminating in overt diabetes. Persistent hyperglycemia also leads to further decrease in beta-cell mass due to a phenomenon called glucotoxicity. Glucotoxicity often leads to beta-cell apoptosis as observed in the pancreas of type 2 diabetic patients, where neogenesis or beta-cell replication failed to compensate for the loss of cells.

Another hallmark feature of T2D is insulin resistance. Insulin resistance is a condition in which the peripheral tissues, namely skeletal muscles, liver and adipose tissue, which are highly insulin responsive under normal conditions, become resistant to insulin, leading to delayed clearance of glucose from the circulation. Insulin normally regulates fuel

homeostasis by promoting the uptake of glucose by the peripheral tissues, inhibiting the release of stored lipids from the adipose tissue and inhibiting hepatic glucose production (32-36). One of the main factors contributing to insulin resistance is obesity. Obesity due to oversupply of nutrients and physical inactivity is strongly correlated to T2D. Under normal circumstances, excessive circulating glucose is taken up mainly (80%) by the skeletal muscles and stored as glycogen. It is therefore widely accepted that skeletal muscles are one of the major sites of insulin resistance in T2D, although liver and adipose tissue could also become equally resistant to insulin. Insulin stimulated glucose uptake by skeletal muscles and adipose tissue is a well-studied signal transduction pathway in humans and rodents. In healthy individuals, in response to rise in circulating glucose, insulin is secreted and released into the blood stream from the pancreatic beta-cells. Binding of insulin to its receptor on the muscle plasma membrane, signals the translocation of the muscle glucose transporter, GLUT 4, to the surface of the plasma membrane, facilitating the uptake and transport of glucose into the muscle cells. However, a number of studies have reported that the insulin-signalling cascade that is critical for the transport of glucose into the muscles cells is impaired under insulin-resistant state. In addition, fatty acids are also known to cause insulin resistance in humans and rodents, although the mechanism underlying lipid-mediated insulin resistance is not completely understood. Fatty acids are generally stored as triglycerides in the adipose tissue. However, in obese individuals, due to oversupply of nutrients, triglycerides accumulate ectopically in tissues such as the muscles, the liver and the heart. In skeletal muscles, excessive accumulation of triglycerides interferes with insulin signalling and translocation of GLUT 4 to the plasma membrane, thereby impairing the intracellular transport of glucose and rendering the muscles resistant to insulin. Fatty liver, a condition that is often diagnosed in insulin-resistant individuals, the liver becomes largely resistant to the actions of insulin, leading to its inability to inhibit gluconeogenesis. In addition, adipose tissue also releases hormones and cytokines, which are also believed to contribute to insulin resistance in animals (26). It is therefore widely accepted that perturbations in both lipid and glucose homeostasis along with beta-cell dysfunction leads to chronic hyperglycemia manifesting in T2D.

Despite the remarkable advancements made in understanding the molecular, genetic and biochemical factors that cause beta-cell failure and insulin resistance in T2D, the underlying mechanisms are still not completely understood. It is generally accepted that T2D is a polygenic disorder in which a complex interplay of genetic and environmental factors contribute to the pathophysiological abnormalities presented such as reduction in beta-cell mass, insulin secretion, insulin action and obesity. Through traditional linkage analysis and genome wide association studies (GWAS), approximately 25 genetic loci harbouring common variants that were related to T2D were discovered (37). Unlike T1D, where the genetic risk is mostly associated with the human leucocyte antigen (HLA) region of the major histocompatibility complex (MHC) on chromosome 6, the genetic component of T2D risk is not concentrated in one specific region and the pathophysiological abnormalities appear to be the result of the interaction of multiple genes scattered all across the genome (38). The role of environmental factors such as obesity, sedentary life style, small or large birth weight and stress, on the development of diabetes has been well studied and well established. However, it is important to understand that even with the same environmental exposures, some people are more susceptible to developing diabetes than others, and this increased risk appears to be genetic in nature.

Type 2 diabetes management, like the disease itself, is multifactorial. Obesity and sedentary life style being the root cause of T2D, life style interventions designed to impact physical activity and food intake are critical to diabetes management. From time and time, research has shown that maintaining an appropriate and stable body weight and body mass index (BMI) is critical to improve glycemic control and decrease cardiovascular diseases. Even a modest weight loss of 5 -10 % has shown to improve glycemic control dramatically in obese people with T2D (39,40). In addition to life style changes, pharmacological interventions are used to manage hyperglycemia in type 2 diabetic patients (41,42). Medications to improve insulin secretion, such as sulfonylureass and meglitinides, those that improve insulin sensitivity such as metformin and thiozolidinedions and those that can slow down intestinal carbohydrate metabolism such as the α-glucosidase inhibitors are some of the different

classes of drugs that are used for the management of hyperglycemia in T2D. However, due to progressive beta-cell dysfunction that is characteristic of T2D, insulin replacement therapy is ultimately required to sustain glucose homeostasis in type 2 diabetic patients.

1.4 Maintenance of Glucose and Energy Homeostasis

Regulation of glucose and energy homeostasis requires the release of appropriate peptide signals from the pancreatic islets, the adipose tissue, the stomach and the gut. These peptide signals interact with the brain and ensure a perfect regulation of energy homeostasis and the brain in turn influences the secretion of these peptides. The human body requires the blood glucose level to be maintained in a narrow physiological range of 70 - 100 mg/dL (equivalent to 3.8 - 5.5 mM). The tight regulation of glucose homeostasis is achieved not only by the actions of insulin, but by the opposing actions of both insulin and glucagon. In response to the postprandial rise in circulating glucose, insulin dampens the rise by promoting the uptake of glucose through peripheral tissues and by suppressing the production of glucose from the liver. On the other hand, glucagon prevents a critical drop in the blood sugar level (hypoglyceamia) in between meals or during fasting by promoting glycogenolysis in the liver and mobilizing glucose from stores inside our body (43,44). Thus, the interplay between insulin and glucagon keeps the blood glucose concentration constant throughout the day.

Although insulin is the major regulator of energy reserves in our body by promoting the uptake and storage of glucose in the form of glycogen and triglycerides, as mentioned in the beginning, the brain, the gut and the adipose tissue also play a critical role in maintaining glucose and energy homeostasis. The key to maintaining energy homeostasis is the underlying basic principle that food intake should match energy expenditure. Any deviation from this basic concept, such as an imbalance between intake and energy expenditure, results in either weight gain or weight loss in humans and rodents (45). The ability of the brain to regulate energy homeostasis depends on its capacity to sense insulin and leptin (46,47). Leptin is a hormone that is secreted by the adipocytes and acts on the brain to regulate food intake and body weight. Both insulin and leptin are commonly known as adiposity signals because they are secreted in direct response to the body fat. The brain has both insulin and

leptin receptors located in several regions, however, they are most abundantly present in the hypothalamus. In response to the postprandial increases in insulin and leptin levels in the brain, the brain sends signals to the liver via the autonomic nervous system to reduce the hepatic glucose production. The importance of insulin and leptin signalling in the brain was supported by several studies. Administering insulin or leptin directly to the brain reduces food intake and weight gain, whereas reductions in either insulin or leptin within the brain, have an opposite effect (48). The brain also signals the gut to secrete duodenal peptides such as, cholecystokinin (CCK) and peptide YY to decrease food intake. Peptide hormones such as those secreted from the intestinal cells like glucagon-like-peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), commonly known as the incretins, in addition to coordinating digestion and absorption of nutrients, also augment the post-prandial increase in insulin secretion and decreases food intake. The gut hormones are generally secreted proportional to the quantity and quality of calories consumed and function as satiety signals to the brain to limit meal size. Ghrelin, secreted from the P/D1 cells of the fundus of the stomach and the epsilon cells of the pancreas is the only hunger-stimulating hormone, stimulating feeding and secretion of growth hormones. Ghrelin stimulates appetite and induces a positive energy balance leading to body weight gain. Leptin and ghrelin are complementary in their action, reflecting acute and chronic changes in energy balance (49,50). Thus, in general, if the brain senses a caloric shortage or surplus, it generates responses that alter food intake, nutrient storage and physiological functions such as hepatic glucose production, adiposity and thermogenesis. Maintenance of glucose, lipid and energy homeostasis in our body is largely dependent on the ability of brain to interpret and integrate neuronal and hormonal inputs. Disruption or failure of any of the key components of brain-gut-endocrine-adipose tissue axis will lead to pathophysiological abnormalities resulting in metabolic disorders such as obesity and type 2 diabetes (Figure 1).

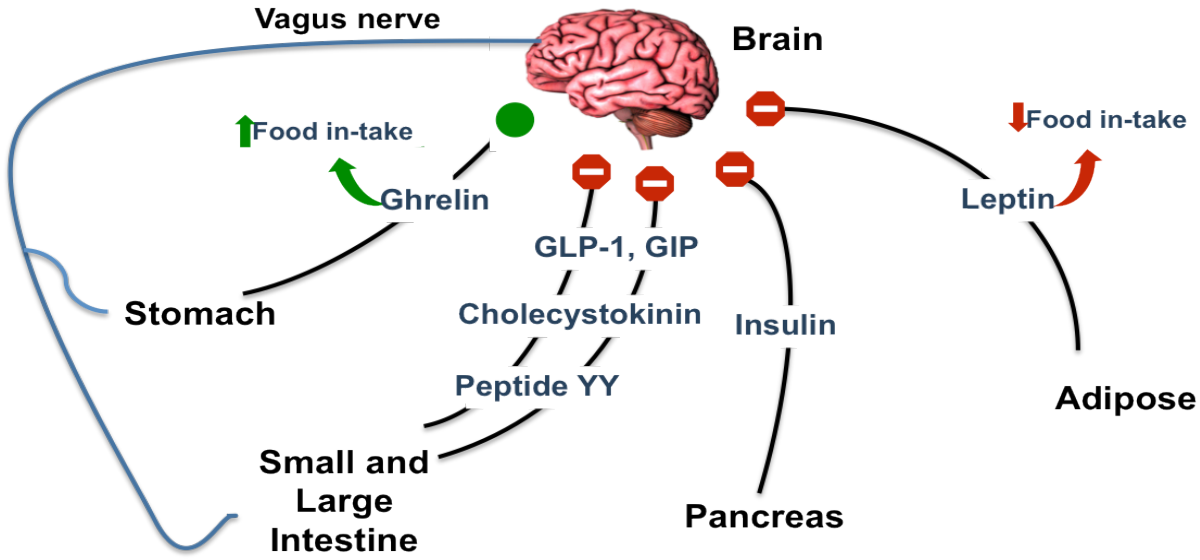

Figure 1: Role of the brain and the peripheral hormones in the regulation of food intake and energy expenditure: The brain integrates both the neuronal and hormonal inputs to determine food intake and energy expenditure. Adiposity signals (long term) such as insulin (pancreas) and leptin (adipose tissue) interact with the arcuate nucleus of hypothalamus decreasing food intake and increasing energy expenditure. Satiety signals (short term) such as the GLP-1, GIP, Cholecystokinin, Peptide YY (Small and large intestine) generated during meals, provide the brain with information such as mechanical (e.g., stomach stretch, volume) and chemical properties of the ingested food, nutritional status and inhibiting food intake. Ghrelin is the only hunger-stimulating hormone, stimulating food intake and secretion of growth hormones.

1.5 Glucose-Stimulated Insulin Secretion (GSIS) - The K_{ATP} CHANNEL Dependent Model

Pancreatic beta-cells secrete the hormone insulin in direct proportion to the concentration of glucose in the circulation. The understanding that beta-cells are inherently designed for the secretion of insulin and that metabolism of glucose generates signals necessary for insulin secretion came from three critical studies which showed that 1) beta-cells only respond to glucose and not to its non-metabolizable counterpart, 2-deoxyglucose 2) beta-cells express non-insulin dependent glucose transporter 2 (GLUT 2), whereas tissues such as the liver and

the skeletal muscles, expresses insulin-dependent glucose transporter 3 (GLUT 3). In beta-cells, glucokinase (GK), the glucose phosphorylating enzyme, is expressed in the physiological concentration of glucose unlike in other tissues such as liver where, the expression is induced only after feeding (51-53). These early discoveries provided ample evidence to show that beta-cells are specialized to secrete insulin for the maintenance of glucose homeostasis.

Insulin secretion from the beta-cell occurs in a bi-phasic manner with a first acute phase happening within 10 minutes after a glucose load. The second more sustained phase of secretion, reaches a plateau very quickly as seen in mice or more gradually as seen in rats and humans (54). Although the mechanisms that control GSIS are not completely understood, the generally accepted model holds that glucose enters beta-cells via the low affinity, high capacity glucose transporter, GLUT 2. Glucose is then phosphorylated by GK (hexokinase 4), which is the first and the rate-limiting step in glycolysis (55). The glycolytic end product pyruvate then enters the tricarboxylic acid cycle (TCA), where it is converted to oxaloacetate (OA) and acetyl CoA. OA and acetyl CoA combine to form citrate and subsequent intermediates of the TCA cycle. Oxidation of these TCA cycle intermediates leads to the generation of reducing equivalents, NADH and $FADH_2$. During the oxidative phosphorylation that follows the oxidation of the TCA intermediates, both NADH and $FADH_2$ act as electron donors and the electrons are passed from electron donors to electron accepters such as oxygen, in a series of redox reactions that happens in the inner mitochondrial membrane. These redox reactions generate a proton gradient across the inner mitochondrial membrane and the protons re-enter the mitochondrial matrix, down their electrochemical gradient, powering the ATP synthase and the energy is used to generate ATP, leading to an increase in ATP production. The subsequent rise in the cytosolic ATP/ADP levels promotes the closure of the ATP-sensitive potassium channels (K_{ATP} channels), causing beta-cell membrane depolarization and activation of voltage-dependent Ca^{2+} channels (VDCC). The opening of VDCCs facilitates influx of extracellular Ca^{2+}, leading to a rise in the cytosolic Ca^{2+} levels, which then triggers the exocytosis of insulin-

containing secretory granules. This pathway of insulin secretion is popularly known as the triggering pathway or the K_{ATP} channel-dependent pathway and is critical for the first acute phase of insulin release, where exocytosis of insulin granules occur from the "readily releasable pool" docked at the beta-cell plasma membrane (54,56-61). However, in the second and more sustained phase of insulin secretion, in addition to the requirement of K_{ATP} channel dependent triggering pathway as an initiating event, the K_{ATP} channel-independent events seem to be necessary for the amplification of GSIS.

1.6 K_{ATP} Channel Independent Pathway of Insulin Secretion

The K_{ATP} channel-independent pathway, also known as the amplifying pathway, is complementary to the triggering pathway and is critical for the sustained secretion of insulin. Important support for the K_{ATP} channel-independent pathway of GSIS comes from studies that showed the ability of glucose to cause a significant increase in insulin secretion in conditions where K_{ATP} channels are held open by application of diazoxide or high K^+ or in animals lacking functional K_{ATP} channels (62-64). Islets from mice lacking the sulfonylurea receptor-1 (SUR1), a subunit of the K_{ATP} channel, retain the ability to secrete insulin and these mice are shown to have relatively normal glucose homeostasis. In addition to this, research has also shown that mitochondrial metabolism of glucose generates signals other than changes in the ATP/ADP ratio that are important for normal insulin secretion. Several molecules, including glutamate, malonyl-CoA/LC-CoA and NADPH, have been proposed as candidate coupling factors in GSIS (65-69) (Figure 2).

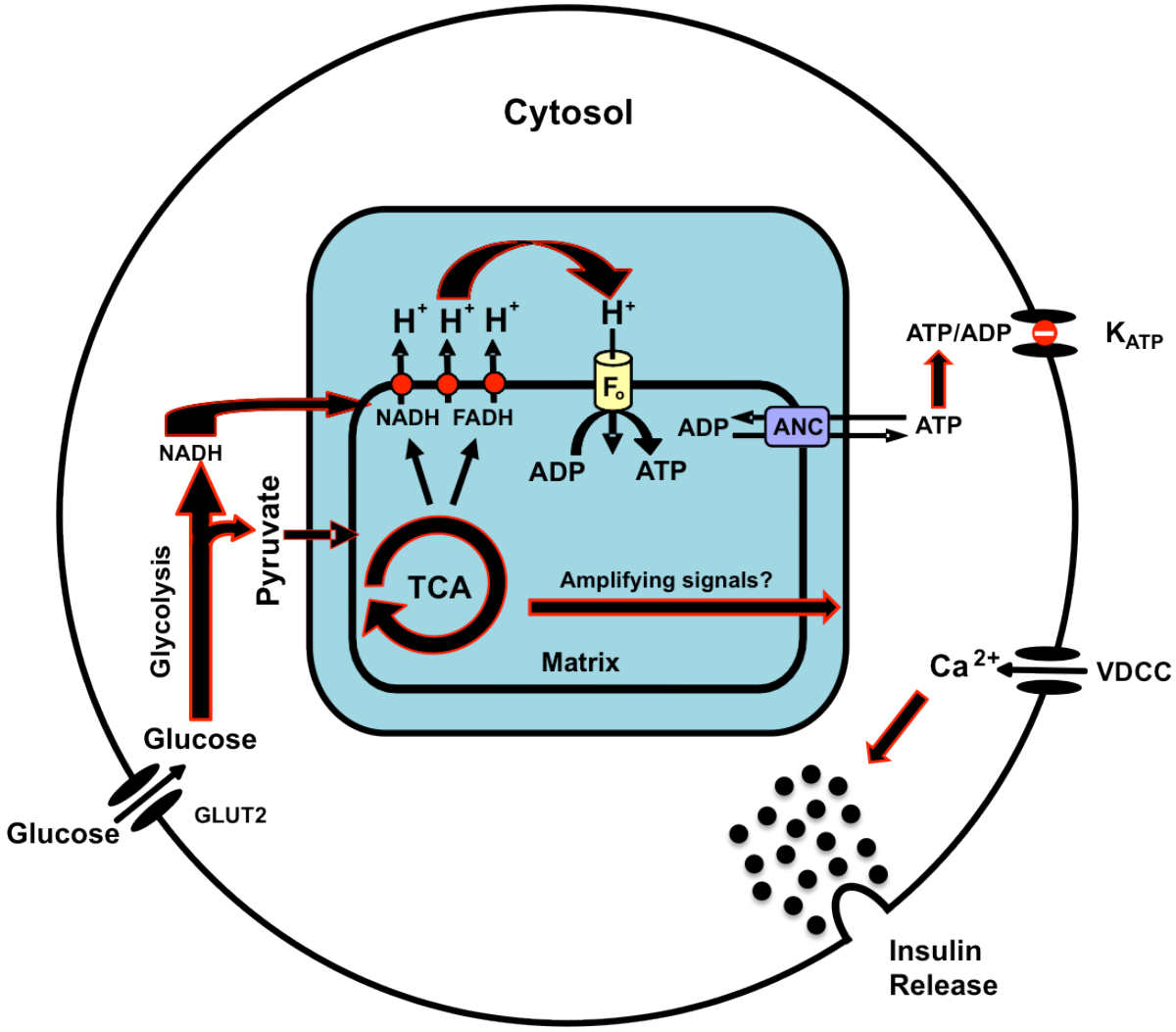

Figure 2: Glucose-stimulated insulin secretion (GSIS) from pancreatic beta-cells. Glucose equilibrates across the plasma membrane through glucose transporter, GLUT2, which initiates glycolysis. Pyruvate produced by glycolysis preferentially enters the mitochondria and is metabolized in the TCA cycle, producing reducing equivalents in the form of NADH and $FADH_2$. The transfer of electrons from these reducing equivalents through the mitochondrial electron transport chain is coupled with the pumping of protons from the mitochondrial matrix to the inter membrane space, leading to the generation of ATP. ATP is transferred to the cytosol through adenine nucleotide carrier (ANC), raising the ATP/ADP ratio. This results in the closure of the ATP sensitive K+ channels (K_{ATP}), which in turn leads to membrane depolarization, opening of the voltage-sensitive Ca^{2+} channels, promoting calcium entry and increase in cytoplasmic Ca^{2+} leading to exocytosis of insulin granules. Glucose also generates amplifying signals other than ATP, which play a significant role in the secretion of insulin from pancreatic beta-cells.

1.6.1 Role of Anaplerosis in GSIS

Among the several potential pathways that play a critical role in supporting the K_{ATP} channel independent pathway of insulin release, the role of anaplerosis deserves special mention. The metabolic fate of pyruvate in the mitochondria has been well studied by several groups. When glucose is readily available, pyruvate enters the TCA cycle in roughly equal proportions via the oxidative and anaplerotic pathways (70-72). The oxidative entry of pyruvate is mediated by the enzyme pyruvate dehydrogenase (PDH), which decarboxylates pyruvate to acetyl CoA. However, the anaplerotic entry of pyruvate is mediated by pyruvate carboxylase (PC), which carboxylates pyruvate to oxaloacetate (OAA). The PC-mediated anaplerosis or replenishment of OAA in the TCA cycle is extremely important for GSIS, as it not only increases TCA flux, but also facilitates the escape of other TCA intermediates from the mitochondria through a process called cataplerosis. These TCA intermediates then serve as a substrate for the generation of cytosolic coupling factors that augment insulin secretion. Several lines of evidence strengthen the role of PC and anaplerosis in beta-cell insulin secretion (70,73,74). 1) PC is highly expressed in beta-cells and 40-50% of the pyruvate entering the mitochondrion at stimulatory glucose concentrations is directed through the PC-mediated anaplerotic pathway. Unlike hepatocytes, where PC is required to promote gluconeogenesis through phospho enol pyruvate carboxykinase (PEPCK), beta-cells lack the expression of PEPCK and express low amounts of fatty acid synthase (FAS) suggesting low gluconeogenesis and lipogenesis. This suggests that PC mediated anaplerosis is important in beta-cells and it mediates a different function as opposed to its primary role in the liver which is gluconeogenesis and lipogenesis. 2) Only 25% of the glucose-carbon entering the TCA cycle via PC is directed to protein synthesis 3) the capacity of various glucose responsive INS-1 derived beta-cell lines for GSIS is tightly correlated to PC-mediated anaplerotic influx of substrates to the TCA cycle and not to PDH-mediated decarboxylation. Thus, PC-mediated anaplerosis and export of TCA intermediates such as citrate from the mitochondria to the cytoplasm is believed to be crucial for generation of anaplerotic signals such as glutamate, malonyl coA/LC-CoA and NADPH to maintain the beta-cell secretory response.

1.6.2 Role of Malonyl-CoA/LC-CoA in GSIS

One of the critically analyzed hypotheses that supports the K_{ATP} channel independent pathway and anaplerosis is the malonyl Coenzyme A/Long Chain Acyl-Coenzyme A (Malonyl CoA/LC-CoA) hypothesis, where glucose induced rise in malonyl CoA/LC-CoA that takes place independently of the K_{ATP} channel function, positively correlates with GSIS (65,66,75). The malonyl CoA/LC-CoA model of GSIS holds that during glucose stimulation, PC-mediated anaplerosis or accumulation of TCA cycle intermediates raises mitochondrial citrate levels, thereby facilitating the escape of citrate from the mitochondria via the citrate/isocitrate carrier (CIC). In the cytosol, citrate serves as a substrate for ATP-dependent citrate lyase (CL) to generate OAA and acetyl CoA. Acetyl CoA then serves as a substrate for acetyl CoA carboxylase (ACC) to produce malonyl CoA, which mediates two important actions in the beta-cells. First, malonyl CoA is an important precursor for the *de novo* generation of free fatty acids (FFA). FFA can then be further processed to LC-CoA by LC-CoA synthetase. Second, malonyl CoA is a potent inhibitor of carnitine palmitoyltransferase 1 (CPT 1), a key regulator (suppressor) of fatty acid (LC-CoA) oxidation. Both of these actions will promote the accumulation of LC-CoA in the cytosol, which has shown to promote GSIS. However, studies have indicated that prevention of glucose-induced rise in malonyl CoA by overexpression of malonyl CoA decarboxylase (MCD) has no impact on GSIS. Furthermore, inhibition of citrate lyase (CL), which plays an important role in the generation of acetyl CoA, a precursor molecule to malonyl CoA, has also been shown to have no impact on GSIS (76), thereby putting into question the exact role of malonyl CoA/LC-CoA in the regulation of GSIS from pancreatic beta-cells.

1.6.3 Role of Pyruvate Cycling in GSIS

Another interesting model that supports the existence of the K_{ATP} channel independent pathway and generation of amplifying signals necessary for insulin secretion is the pyruvate cycling pathway. Pyruvate cycling is the process by which the glycolytic end product pyruvate is replenished in sufficient amounts in the TCA cycle through three different cycles namely the pyruvate-malate, pyruvate-citrate and the pyruvate-isocitrate cycle. Along with

the maintenance of the pyruvate pool in the cytosol, the three pathways generate reducing equivalents such as NADH and NADPH, which may act as potential stimulus-secretion coupling factors critical for GSIS (70,77-79). The relationship between pyruvate cycling and GSIS came to prominence when a study conducted by Lu et al (70) reported that insulin secretion is directly proportional to the activity of pyruvate cycling. Using ^{13}C-NMR, the metabolic fate of pyruvate was analyzed in a set of INS-1 derived clonal cells with robust or poor GSIS. The results showed that the capacity of various lines of INS-1 clonal cells for GSIS was tightly correlated with PC-catalyzed anaplerotic influx into the TCA cycle and pyruvate cycling. Also, stimulation of pyruvate cycling by the addition of dimethyl malate, a membrane permeable methyl ester of malate, increases pyruvate cycling and GSIS, whereas, addition of phenylacetic acid (PAA), an inhibitor of PC decreases GSIS. In contrast, Jenssen et al reported that siRNA mediated inhibition of PC neither affects GSIS in 832/13 cells and rat islets nor does it affect glucose induced increments in pyruvate cycling. However, despite the suppression of PC protein levels by 60% in 832/13 cells, flux through PC at high glucose was decreased by only 20%, suggesting an increase in PC specific activity (80). In addition, Jenssen et al also reported that siRNA mediated suppression of PC increased the levels of acetyl CoA, which is an allosteric activator of PC. This may have aided in maintaining PC activity intact in an attempt to prevent impairment in anaplerosis, pyruvate cycling and GSIS. Thus, PC mediated anaplerosis and pyruvate cycling is believed to play a key role in GSIS.

In an attempt to understand the identity of the pyruvate cycling pathway that is most critical for the generation of signalling molecules necessary for GSIS, recent studies have focused on uncovering the mechanisms that govern each of the pyruvate cycling pathway. In the pyruvate-malate cycle, oxaloacetate (OAA) is converted to malate via malate dehydrogenase (MDH), and subsequently, malate can be reconverted to pyruvate by NADP-dependent cytosolic malic enzyme (MEc) or NAD- dependent mitochondrial malic enzyme (MEm). Several lines of evidence suggest that this pathway is essential in the generation of NADH, which is a by-product of the MEm-catalyzed reaction. Studies carried out by Eto et al shows that NADH produced by cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

must be shuttled into the mitochondria for ATP production (81). When islets from GAPDH knock-out mice were treated with aminooxyacetate, an inhibitor that blocks the malate-aspartate NADH shuttle, it led to the inhibition of glucose-induced increases in glucose oxidation, ATP production and insulin secretion. Others have also shown the importance of NADH generation through MEm, thus providing further proof that pyruvate-malate through MEm is important for GSIS (82). On the contrary, it has been reported that mouse islets with low MEc enzyme activity and MOD 1 mice, which lack the expression of MEc, have normal glucose homeostasis suggesting that pyruvate-malate cycling through MEc may not be universally correlated with GSIS (83,84). Further weakening the association of pyruvate-malate cycling with GSIS, Ronnebaum et al has shown that although siRNA-mediated inhibition of MEc in 832/13 cells impaired GSIS, these effects were not due to changes in pyruvate cycling activity and inhibition of both MEc and MEm failed to impair GSIS in primary rat islets (85). Thus, although the pyruvate-malate cycle has been shown to play a role in pyruvate cycling, a definitive role for this cycle in GSIS is still being debated.

In the pyruvate-citrate cycle, citrate formed from the TCA cycle is exported from the mitochondria to cytosol through the citrate/isocitrate carrier, where it is cleaved to oxaloacetate (OAA) and acetyl CoA by citrate lyase (CL). Cytosolic OAA is then converted back to pyruvate via a two-step process; 1) OAA is converted to malate by malate-dehydrogenase (MDH), 2) malate is converted to pyruvate by the cytosolic malic enzyme (MEc). Support for the pyruvate-citrate cycle in GSIS comes from several studies (86-88). It is generally believed that export of citrate from the mitochondria to the cytosol through CIC leads to an increase of citrate in the cytosol, which is needed for de novo lipogenesis and for the generation of long-chain acyl CoA, a factor that is thought to act a stimulus-secretion coupler and both of these correlate well with GSIS. Studies have also shown that both pharmacological and genetic inhibition of CIC, leads to impairment in GSIS coupled with decrease in cytosolic citrate and de novo lipogenesis. However, the importance of the pyruvate-citrate cycle in GSIS is also somewhat controversial, as inhibition of CL by hydroxyl citrate has yielded different effects on GSIS: one group showing inhibition of GSIS

(89) and the other showing no effects on GSIS (76). Thus, although the export of citrate to the cytosol plays a critical role in supporting GSIS, the exact role of CL in maintaining pyruvate-citrate cycling and GSIS is still not completely understood.

In the pyruvate-isocitrate cycle, both citrate and isocitrate are exported from the mitochondria to the cytosol where cytosolic aconitase converts citrate to isocitrate and cytosolic isocitrate is converted to alphaketoglutarate (α-KG) through cytosolic isocitrate dehydrogenase (ICDc). α-KG may directly stimulate GSIS (90) or it re-enters the mitochondria where it is exchanged for malate, which is then transported to the cytosol and converted back to pyruvate through the MEc. α-KG that enters the mitochondria is converted ultimately to malate and malate to pyruvate through mitochondrial malic enzyme (MEm). The relevance of pyruvate-isocitrate cycling in GSIS was supported by a study where, siRNA mediated inhibition of ICDc lead to inhibition of GSIS in both 832/13 cells and primary rat islets coupled with decrease in pyruvate cycling activity and production of NADPH (91). Based on this evidence, it is believed that, among the three pyruvate cycling pathways, the pyruvate-isocitrate pathway is the most critical one for the maintenance of GSIS from beta-cells (Figure 3).

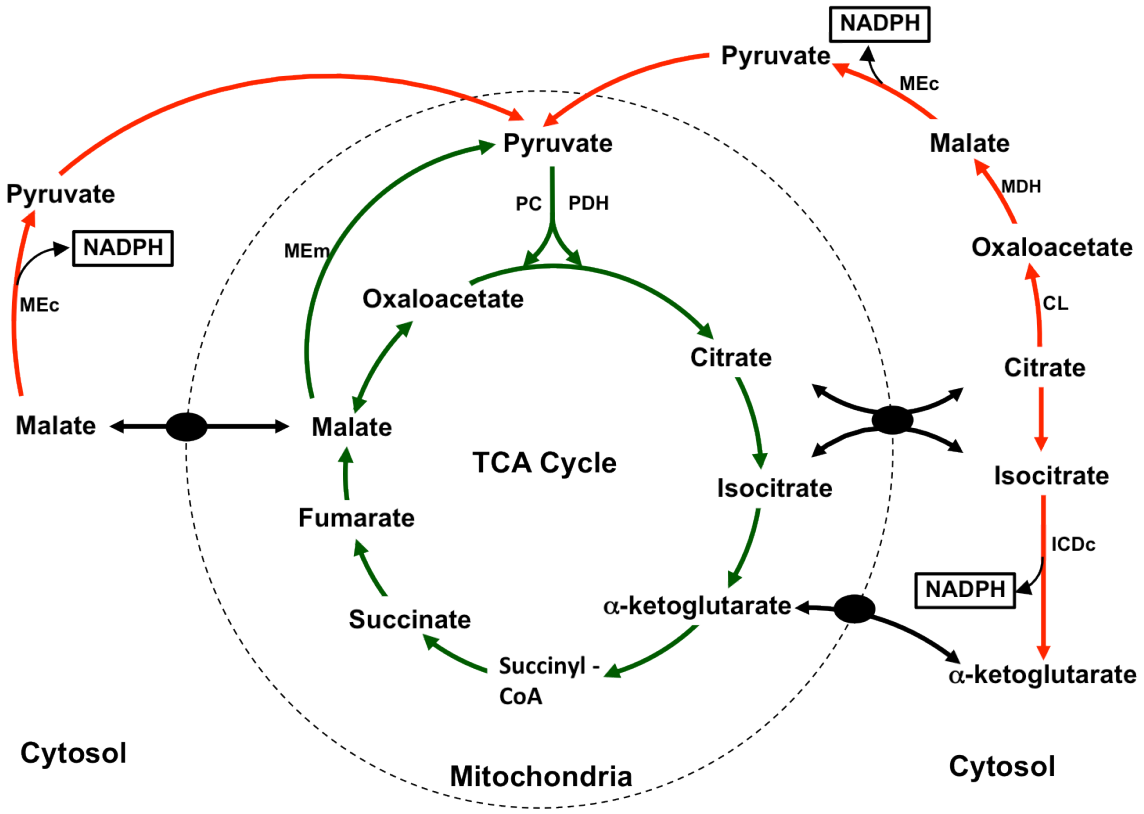

Figure 3: Pyruvate cycling and production of NADPH: The anaplerotic substrate pyruvate is replenished in sufficient amounts through three different pathways. In the pyruvate-malate pathway, pyruvate is converted by the anaplerotic enzyme pyruvate carboxylase (PC) to oxaloacetate. Oxaloacetate is then converted to malate, which exits the mitochondria. Malate can then either be recycled to pyruvate via the mitochondrial, NAD-dependent form of malic enzyme (MEm) or can be transported to the cytosol via the dicarboxylate carrier (DIC). In the cytosol, malate can be reconverted to pyruvate by the cytosolic NADP-dependent form of malic enzyme (MEc). In the pyruvate-citrate cycle, pyruvate enters the TCA cycle via PC. The oxaloacetate generated then condenses with acetyl-CoA to form citrate and isocitrate, which can exit the mitochondria via the citrate/isocitrate carrier (CIC). In the cytosol, citrate is cleaved to oxaloacetate and acetyl-CoA by ATP-citrate lyase (CL). Oxaloacetate is recycled to pyruvate via conversion to malate and engagement with the mitochondrial (MEm) or cytosolic (MEc) malic enzyme to reform pyruvate. In the pyruvate-isocitrate cycle, the cycle is again initiated by the conversion of pyruvate to oxaloacetate by PC. As in the pyruvate/citrate cycle, citrate and isocitrate leave the mitochondria via CIC. Citrate is then converted to isocitrate by cytosolic aconitase, and isocitrate can then be converted to α-Ketoglutarate (α-KG) by cytosolic, NADP-dependent isocitrate dehydrogenase (ICDc). α-KG can then serve either as a direct signal for insulin secretion or it is recycled back to pyruvate by one of several mitochondrial or cytosolic pathways that remain to be defined. An important by-product of all three pyruvate-cycling pathways is the cytosolic NADPH, which is potentially considered to be an insulin secretagogue.

1.6.4 NADPH as a Stimulus-Secretion Coupling Factor in GSIS

An important stimulus-secretion coupling factor in GSIS that has received a lot of attention in the recent years is the pyridine nucleotide, NADPH. Examination of mitochondrial glucose metabolism in clonal beta-cell lines with various degrees of glucose responsiveness demonstrated a strong correlation between pyruvate cycling and glucose competence (70). This strong correlation may be attributed to the generation of cytosolic NADPH, which is produced by either $NADP^+$-dependent malic enzyme (MEc) in the pyruvate/malate and pyruvate/citrate cycle or $NADP^+$-dependent isocitrate dehydrogenase (ICDc) in the pyruvate/isocitrate cycle. A role for cytosolic NADPH in GSIS is supported by several studies. It has been shown that NADPH is an expected by-product of all the three proposed pyruvate cycling pathways (80,85,88,91). Studies have also shown that the $NADPH/NADP^+$ ratio increases in direct proportion to GSIS in rodent islets and several beta-cell lines, whereas such a linear relationship does not seem to exist for the $NADH/NAD^+$ ratio (67,91). The most exciting piece of evidence in support of NADPH was provided by Ivarsson et al, where they showed that addition of NADPH augmented insulin release from patch-clamped beta-cells and this stimulatory effect could be reversed by the addition of $NADP^+$, suggesting that the $NADPH/NADP^+$ ratio is a relevant signal in GSIS (67). Another convincing piece of evidence in favor of NAPDH and why it may be critical for GSIS is based on studies that showed that NADPH binds to voltage-gated potassium channels known as K_V channels, causing the inactivation of these channels (92,93). K_V channels are associated with repolarization of the plasma membrane and it is believed that binding of NADPH will assist in keeping the channels inactive and keeps the plasma membrane in a depolarized state sufficient to sustain insulin secretion. Accordingly, it has been reported that adenovirus mediated expression of truncated Kv2.1 subunit, specifically eliminating Kv2 family currents, enhanced GSIS from rat islets by 60% (94) and Kv2.1 null mice have elevated serum insulin levels (95).

1.7 ARNT/HIF-1β: Structure and Function

Aryl hydrocarbon receptor nuclear translocator (ARNT)/hypoxia inducible factor -1β (HIF-1β) is a member of the basic helix-loop-helix (bHLH)-periodicity/ARNT/Single minded (PAS) family of transcription factors, required for a wide variety of biological processes such as organogenesis, neural development and cellular responses to hypoxia and environmental pollutants (96). The gene that encodes ARNT/HIF-1β was mapped on human chromosome 1q21 and mouse chromosome 3 and seems to be well conserved on an evolutionary scale (97). Human ARNT/HIF-1β gene is ~ 65 kilobases long with 22 exons and shares 89% sequence homology with the mouse ARNT/HIF-1β gene. Compared to other members of the bHLH-PAS family of transcription factors, the exon/intron arrangement in mouse ARNT/HIF-1β gene is different, where the gene was found to be TATA-less with multiple transcriptional start sites. The presence of multiple transcriptional start sites and GC-rich sequences in the promoter region are often considered as characteristic of "housekeeping" genes that have a TATA-less promoter (98).

Earlier data regarding ARNT/HIF-1β structure was mostly derived from cultured wild type and mutant mouse hepatoma cell lines (99). It is generally believed that ARNT/HIF-1β is a nuclear protein, however, studies have also reported that ARNT/HIF-1β is localized to both the cytoplasm and the nucleus of palatal epithelial cells (100). The 94 kD human ARNT/HIF-1β protein has a characteristic N-terminal bHLH motif required for DNA binding, the central PAS domains (PAS A and PAS B) that facilitates heterodimerization and a C-terminal domain that allows the recruitment of transcriptional co-regulators such as CBP/P300 and is 789 amino acids long (101,102) (Figure 4). Recent evidence suggests that the PAS domain may also provide an additional binding site for co-activators, there by recruiting them in a step necessary for transactivation of target genes (103). One of the salient features of ARNT/HIF-1β is that it acts as an obligate binding partner for most bHLH-PAS family transcription factors and binds specific DNA sequences in the regulatory regions of target genes. The half-site for ARNT/HIF-1β is on the 3' side of the 5'-GTG-3' recognition

sequence. The sequence of the other half of the binding site depends upon the identity of the ARNT/HIF-1β dimerization partner (104). Although DNA binding of ARNT/HIF-1β is mostly mediated by its basic region, it is suggested that the PAS region may also be involved. Dimerization between ARNT/HIF-1β and other bHLH-PAS proteins is mediated by their bHLH and PAS regions (105,106). ARNT/HIF-1β was originally identified by Reyes et al in 1992 as a factor required for the activity of the aryl hydrocarbon receptor (AhR) (107). The AhR- ARNT/HIF-1β complex induces the expression of xenobiotic enzymes in response to environmental pollutants such as dioxins (108). ARNT/HIF-1β was later also identified as the β-subunit of the HIF-1 transcription factor, required for the induction of genes necessary for cellular response to hypoxia (102). Some of the well-studied members of the bHLH-PAS family of transcription factors in mammals include AhR, HIF-1α, HIF-2α and HIF-3α.

ARNT/HIF-1β has two homologues, namely ARNT 2 and ARNT 3 (also known as bMAL, MOP3, ARNTL1) (109-112). Despite the high sequence conservation within their DNA binding and dimerization domains and with very similar DNA recognition specificities, the homologues are functionally non-redundant and biologically essential. Gene expression analysis reveals that ARNT/HIF-1β has a ubiquitous expression pattern while ARNT homologues seem to be mainly restricted to the brain and the kidney (113). ARNT/HIF-1β is also known to have several splice variants, which were shown to arise from alternate splicing of the mRNA. Isoforms with deletions at exon 5, 3’ end of exon 6 or 5’ end of exon 11 or with an insertion at 5’ end of exon 20 were discovered in rat suggesting an intricate regulatory system (114).

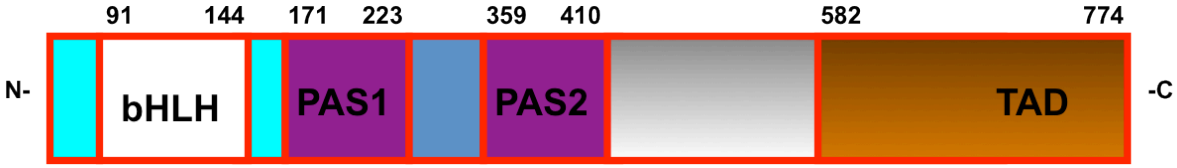

Figure 4: Schematic representation of the human ARNT/HIF-1β protein domains: ARNT/HIF-1β is a member of the basic-helix-loop-helix (bHLH) family of transcription factors with a characteristic N-terminal bHLH domain facilitating DNA binding, two Per/ARNT/SIM (PAS) domains which helps in heterodimerization with partners such as HIF-1α, HIF-2α and AhR and a C-terminal transactivation domain (TAD), facilitating the recruitment of transcriptional co-activators. The boxes represent the length of each domain and the numbers represent the amino acids.

The bHLH-PAS family of proteins usually function by binding the DNA in a dimeric form. Most bHLH-PAS transcription factors form heterodimers with other members of the family to activate gene transcription. Widely studied and well-accepted heterodimeric partnerships of ARNT/HIF-1β include its association with AhR following ligand activation of AhR, assisting in metabolism of xenobiotics, with HIF-1α subunits under low oxygen conditions, assisting with gene expression under hypoxia and with SIM proteins, assisting in neural development (115-117). In addition to forming heterodimers, ARNT/HIF-1β has been shown to form homodimers and regulate genes that typically contain the palindromic E-box (CACGTG) signature in their promoter (118,119). Although, physiological relevance of ARNT/HIF-1β homodimers was not known when they were initially reported in 1995, in 2007, Arpiainen et al., reported that ARNT/HIF-1β homodimers are capable of inducing expression of the mouse Cyp2A5 gene, suggesting the involvement of ARNT/HIF-1β homodimers in xenobiotic metabolism (120).

Despite the extensive research conducted to understand the biological role and physiological relevance of ARNT/HIF-1β in mammalian tissues, the post-translational regulation of ARNT/HIF-1β is largely unknown. Some studies report that ARNT/HIF-1β gene expression and protein levels are significantly increased under hypoxic conditions (96,102,121), suggesting that it plays an important role in the transcriptional response to low oxygen tension. Consistent with this idea, it has been shown that ARNT/HIF-1β is essential for HIF-1α dependent hypoxic induction of vascular endothelial growth factor (VEGF) and the

glycolytic enzymes aldolase A (ALDO) and phosphoglycerate kinase (PGK) in a mouse hepatoma (Hepa 1c1c7) cell line (122,123). However, unlike HIF-1α, which has an oxygen dependent degradation domain (ODDD) and is expressed exclusively under hypoxic conditions, ARNT/HIF-1β protein does not have an ODDD and some studies have reported that it is constitutively expressed in most tissues with little or no response to hypoxia (121,124,125). Other studies have reported that ARNT/HIF-1β protein levels were decreased by hydrogen peroxide (H_2O_2) or reactive oxygen species (ROS) in human cell lines, however, the underlying molecular mechanism is currently unknown (126,127). A few groups have also investigated the phosphorylation status of ARNT/HIF-1β protein in an attempt to understand post-translational regulation of the protein. Generally, protein phosphorylation is thought to modify DNA binding ability, protein-protein interactions as well as translocation to the nucleus. Pongratz et al had shown the importance of AhR - ARNT/HIF-1β phosphorylation for the activation of the heterodimer, thus promoting DNA binding (128). In another study by Berghard et al, it was reported that DNA binding by human and mouse AhR - ARNT/HIF-1β heterodimers requires phosphorylation of both proteins, however, formation of AhR - ARNT/HIF-1β heterodimers requires phosphorylation of only ARNT/HIF-1β protein (129,130). Another group had shown that although human ARNT/HIF-1β is a phosphoprotein, the phosphorylation of serine residues in the PAS region does not modulate AhR - ARNT/HIF-1β or ARNT/HIF-1β - ARNT/HIF-1β-mediated signal transduction (131). Thus, although the exact consequence of ARNT/HIF-1β protein phosphorylation is unknown, it is generally believed that overall phosphorylation status rather than site-specific phosphorylation may be crucial in the regulation of ARNT/HIF-1β mediated signal transduction (131).

1.8 ARNT/HIF-1β in Metabolism of Xenobiotics

In response to ligand activation of AhR following exposure to xenobiotics such as polycyclic aromatic hydrocarbons and halogenated aromatic compounds, AhR, a cytoplasmic protein translocates to the nucleus, where it binds with its heterodimeric binding partner ARNT/HIF-1β (132). Transcriptional induction of enzymes required for the metabolism of xenobiotics is initiated when AhR- ARNT/HIF-1β complex binds to the xenobiotic responsive element

(XRE: TNGCGTG) of target genes such as Cyp1A1, Cyp1B1, and NADP(H):oxidoreductase (NQO1)(133,134). Upon DNA binding, AhR- ARNT/HIF-1β complex like other nuclear receptors, recruits transcriptional co-activators such as CBP/p300, SRC-1, NCoA2/GRIP1/TIF2, and components of ATP-dependent chromatin remodeling complexes, such as Brahma-related gene 1 (BRG-1) and TRIP230. These proteins then interact and modulate the activity of the core transcriptional machinery, as well as modify local chromatin structure, thereby inducing gene expression. Subcellular localization studies and immunohistochemical analysis have revealed that AhR, prior to ligand activation, is localized in the cytoplasm bound to 2 molecules of 90-kDa-heat shock protein (HSP90) (135). Upon ligand activation, AhR transforms, releasing the HSP90, translocates to the nucleus and binds with ARNT/HIF-1β. In fact ARNT/HIF-1β was originally cloned as a factor required for the translocation of AhR from the cytoplasm to the nucleus. Briefly, a cell line expressing a "functional" AhR, but uninducible for Cyp1A1, a cytochrome p450 family member, was transfected with a collection of cDNA constructs. The expression of one of the cDNA construct restored the nuclear translocation of AhR and the expression of Cyp1A1. The product of this gene was therefore named ARNT (Ah receptor nuclear translocator), although we now know that ARNT is not directly involved in the AhR "translocation". Following its cloning in 1992 by the Hankinson group (107), detailed structure and function analysis were carried out on ARNT/HIF-1β protein. These studies have clearly established that the transactivation potential of AhR-ARNT/HIF-1β complex is highly dependent on the availability of ARNT/HIF-1β (108,136). Although, ARNT/HIF-1β alone has no affinity for the XRE, the heterodimerization with AhR, promotes its interaction with the XRE of the target gene (137). Genetic analysis of a mouse hepatoma cell line deficient in ARNT/HIF-1β shows poor response to dioxins, which was restored by transfection of ARNT/HIF-1β cDNA (138). This and more studies indicate that ARNT/HIF-1β is an obligatory binding partner for AhR-mediated transcriptional response to the metabolism of environmental toxins or xenobiotics.

The AhR - ARNT/HIF-1β heterodimer can also impact signal transduction by the estrogen receptor (ER). ARNT/HIF-1β was shown to be a potent co-activator of both ER-α and ER-β-

dependent transcription and the C-terminal domain of ARNT/HIF-1β is believed to be essential for the transcriptional enhancement of ER activity (139,140).

1.9 ARNT/HIF-1β in Hypoxia

Oxygen is the primary substrate for generation of ATP and hence critical for maintaining cellular activities in all multicellular organisms. Hypoxia, a condition where oxygen levels become extremely low in our body, occurs in both physiological and pathophysiological conditions. To combat the adverse effects of hypoxia, our body has inherent and specialized signalling proteins called the hypoxia inducible factors (HIF), which are transcription factors regulating the signal transduction pathway called the hypoxia-signalling pathway. With cloning and characterization of HIF-1α by Semenza et al in 1992 (141), followed by purification and cloning of the components of the HIF-1 protein complex, consisting of HIF-1α and ARNT/HIF-1β in 1995 by the same group (102), the hypoxia signalling pathway became a much celebrated pathway due its biological role in mammalian development and prominence in disease pathology.

The signal transduction pathway induced by hypoxia is well studied and well accepted in mammals (142,143). When cells are exposed to low oxygen conditions, HIF-1α, a cytoplasmic protein that has an oxygen dependent degradation domain (ODDD), translocates to the nucleus where it binds to its heterodimeric binding partner, ARNT/HIF-1β. The HIF-1α - ARNT/HIF-1β transcriptional complex then binds to the hypoxia responsive element (HRE: RCGTG, where the R is either an A or a G) in the promoter region of target genes. Upon binding to the HRE, HIF-1 complex also recruits transcriptional co-activators such as CBP/p300 which then assist in HIF-1 mediated gene transcription.

Regulation of HIF-1α - ARNT/HIF-1β mediated gene transcription under hypoxic conditions is mainly dependent on the availability of HIF-1α, as the protein is normally degraded under normoxic conditions. HIF-1α, under normoxia, is post-translationally regulated by the prolyl hydroxylation of the ODDD (143). The hydroxylated HIF-1α protein is then a target of proteosomal degradation by promoting their interaction with von Hippel-Lindau (VHL)

protein, a component of E3 ubiquitin-ligase multi-protein complex (144). Although, ARNT/HIF-1β is believed to be constitutively expressed and available for binding with HIF-1α under all conditions, studies have shown the absolute requirement of ARNT/HIF-1β for HIF-1α mediated hypoxic activation of gene transcription. Using a mouse hepatoma cell line that lacks functional ARNT/HIF-1β protein, both HIF-1 transcriptional complex formation as well as hypoxic induction of target genes were found to be defective (123,145,146). Taken together, ARNT/HIF-1β has been shown to be absolutely indispensible for HIF-1 DNA binding and transactivation (Figure 5).

The HIF-1α - ARNT/HIF-1β complex is required for the hypoxic induction of target genes such as those that are required for the development of the hematopoietic and vascular system namely vascular endothelial growth factor (VEGF) and erythropoietin (EPO) and those that are required for cell proliferation and differentiation by regulation of enzymes in glucose uptake and metabolism and the mammalian target of rapamycin (mTOR), which regulates protein synthesis in response to the nutritional status of the body (4,147-154).In addition to hypoxia, several non-hypoxic stimuli can also induce HIF transcriptional activity. HIF-1α transcriptional activity has been shown to be promoted by effectors of immune response such as cytokines and those that modify the nuclear factor κB signalling pathway (155-158). Accumulation of HIF-1α protein was shown to be stimulated by the autocrine growth factors such as epithelial growth factor (EGF), fibroblast growth factor 2 (FGF2) and insulin-like growth factor (IGF). In addition, loss of function of tumor suppressors (such as ING4, p53, PTEN and VHL) as well as gain of function of oncogenes (such as AKT, myc, mTOR, PI3K etc) also regulates HIF-1α activity.

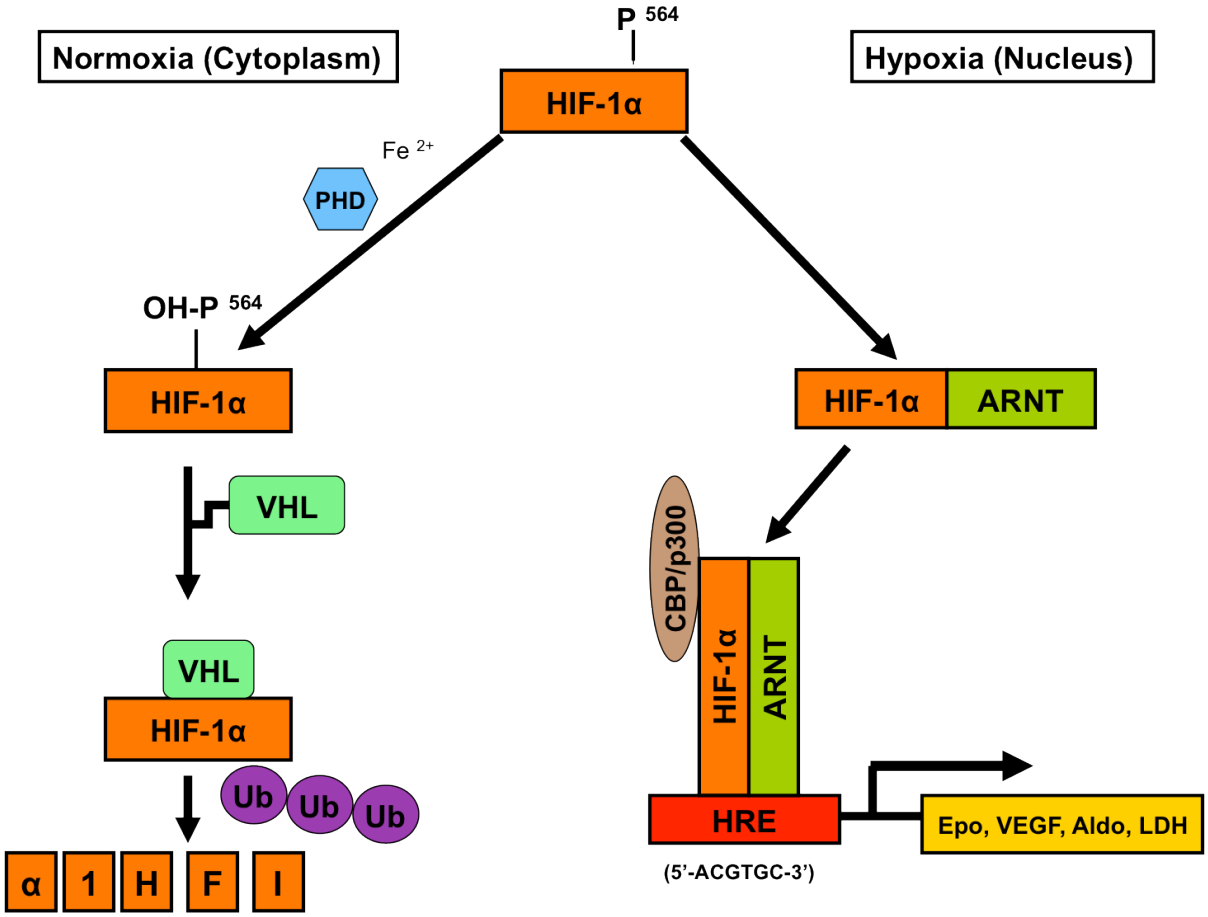

Figure 5: Transcriptional Regulation by ARNT-HIF-1α heterodimeric complex. In normoxia, a specific proline within HIF-1α (P ^{564}) is hydroxylated by O_2- and Fe^{2+}-dependent HIF-1α prolyl hydroxylases (PHD). This modification prepares HIF-1α protein to associate with von Hippel Lindau protein (VHL) for ubiquitination by E3 ubiquitin ligase complex leading to the degradation of HIF-1α protein. In hypoxia, the required oxygen tension for PHD activity is absent. As a result, HIF-1α protein is stable and translocates to the nucleus where it heterodimerizes with ARNT/HIF-1β to form a stable transcriptional complex, which then associates with transcriptional activators such as CBP/p300. This complex subsequently binds to the hypoxia responsive element (HRE) of target genes and activates gene transcription of genes such as erythropoietin (Epo), vascular endothelial growth factor (VEGF), aldolase (Aldo) and lactate dehydrogenase (LDH). Adapted from Fedele et al 2002 (159).

1.10 Lessons from ARNT/HIF-1β Knock-Out Mice

Observations from the ARNT/HIF-1β conditional knock-out mice and whole body knock-out mice have provided us with a wealth of information regarding the functional significance of this transcription factor in mammalian development. By the targeted disruption of the bHLH domain of the murine ARNT/HIF-1β gene, Maltepe et al showed that $ARNT^{-/-}$ embryonic stem cells (ES) failed to activate genes that normally respond to low oxygen tension such as ALDO, PGK and VEGF. $ARNT^{-/-}$ ES cells also failed to respond to a decrease in glucose concentration, indicating it is crucial in response to hypoxia and hypoglycemia. ARNT/HIF-1β seems to be essential for normal embryonic development as $ARNT^{-/-}$ embryos were not viable past day 10.5 and exhibited angiogenic abnormalities similar to those reported for mice lacking VEGF (160,161). More support for the requirement of ARNT/HIF-1β in normal embryonic development came from the Hankinson group who reported that whole body ARNT/HIF-1β knock-out mice were embryonic lethal between 9.5 and 10.5 days of gestation, with abnormalities that included neural tube closure defects, forebrain hypoplasia, delayed rotation of the embryo, placental hemorrhaging, and visceral arch abnormalities. However, the primary cause of lethality was reported to be failure of the embryonic component of the placenta to vascularize, which is related to ARNT/HIF-1β's known role in hypoxic induction of angiogenesis (162). In order to study the role of ARNT/HIF-1β in an intact animal, a conditional knock-out mouse was generated by Tomita et al (163) using the Cre-loxP system, where treatment with polyinosinic-polycytidylic acid was used to induce expression of Cre resulting in complete disruption of the ARNT/HIF-1β gene expression and protein expression in the liver. This study was the first of its kind to show in an intact animal that complete disruption of ARNT/HIF-1β in the liver results in loss of AhR mediated gene activation in the liver. In addition, loss of ARNT/HIF-1β in liver was also shown to affect the expression of HIF-1 mediated expression of target genes such as hemoxygenase and GLUT1. Thus, these earlier *in vivo* studies established the undisputable role of ARNT/HIF-1β in mammalian development as well as in the hypoxia signal transduction pathway.

1.11 ARNT/HIF-1β and Type 2 Diabetes

T2D is a complex metabolic disorder characterized by disturbances in whole body glucose and lipid homeostasis. Regulation of glucose and lipid homeostasis involves proper co-ordination between multiple organs of the body, namely, the pancreatic beta-cells, the liver, the skeletal muscles, the adipose tissue and the brain. Since the discovery of ARNT/HIF-1β in 2005 as a protein required for the normal functioning of pancreatic beta-cells (164), several groups have investigated the role of this transcription factor in the pathogenesis of T2D. Recent research suggest that abnormal expression of ARNT/HIF-1β and its binding partner, HIF-1α can have a negative impact on the major metabolic regulators of the body such as the pancreatic beta-cells, the liver and the adipose tissue, potentially contributing to the pathophysiological abnormalities presented in T2D.

1.11.1 ARNT/HIF-1β and Pancreatic Beta-Cells

In 2005, a study published in Cell reported that ARNT/HIF-1β, a protein with previously unknown functions in pancreatic beta-cells, plays a significant role in mediating beta-cell dysfunction in T2D (164). Genome-wide gene expression profiling of islets obtained from normal and type 2 diabetic patients revealed that expression of ARNT/HIF-1β was reduced by 90% under diabetic conditions. In addition to ARNT/HIF-1β, genes involved in glycolysis and insulin signalling were also found to be down-regulated in diabetic islets. Several enzymes in glycolysis, which were also ARNT/HIF-1β targets, including phosphoglucomutase (PGM), phosphoglucose isomerase (G6PI), phophofructokinase (PFK) and aldolase, were expressed at significantly lower levels in diabetic islets compared to normal islets. Expression of key regulators of the insulin signalling pathway, such as the insulin receptor (IR), insulin receptor substrate 2 (IRS2), and protein kinase B (Akt2) was also found to be down-regulated in diabetic islets. In addition, genes responsible for maturity onset diabetes of the young (MODY), a monogenic form of diabetes, namely, HNF-1α and HNF-4α, were found to be poorly expressed in islets obtained from type 2 diabetic patients. HNF-4α, mutated in MODY1, has been shown to interact with ARNT/HIF-1β possibly providing a connection between the two transcription factors (165).

In order to rule out the possibility that the profound ARNT/HIF-1β down-regulation in pancreatic beta-cells was not caused by the diabetic environment, Gunton and co-workers demonstrated that an identical gene profile was observed in a beta-cell-specific ARNT/HIF-1β knock out mouse (β-ARNT KO) (164). β-ARNT KO mice had reduced expression of genes involved in glycolysis and insulin signalling, similar to those observed in diabetic islets. The mice also exhibited impaired *in vitro* and *in vivo* GSIS and glucose intolerance, with no significant change in beta-cell insulin content and islet mass. Thus, through a series of *in vivo* and *in vitro* experiments in humans and rodents, Gunton et al provided strong and convincing evidence that reduction in ARNT/HIF-1β expression in pancreatic beta-cells has negative consequences in terms of beta-cell function and insulin secretion. However, the extent of ARNT/HIF-1β-mediated regulation of gene transcription seems much more complex than one might expect as it has the potential to bind with multiple partners affecting a multitude of signalling pathways.

Following Gunton's discovery of ARNT/HIF-1β as the most down-regulated gene in diabetic islets, a few groups have focused their attention to identify the upstream regulators of the transcription factor in pancreatic beta-cells. Dror et al identified presenilin, a protein that was shown to be involved in the regulation of beta-cell survival, in calcium homeostasis and response to hypoglycemia in neurons, as an upstream regulator of ARNT/HIF-1β (166-168). In another study, based on chromatin immunoprecipitation and high density oligonucleotide arrays, carbohydrate responsive element binding protein (ChREBP), a transcription factor involved in carbohydrate metabolism in the liver and pancreatic beta-cells, was identified as a negative regulator of ARNT/HIF-1β. Their results suggested that ChREBP-mediated repression of the HIF complex might contribute to beta-cell dysfunction induced by glucotoxicity (169). However, in 2010, Gunton's group presented a substantial amount of evidence once again, where HIF-1α, the heterodimeric binding partner of ARNT/HIF-1β was identified as an upstream regulator of ARNT/HIF-1β (170). Similar to ARNT/HIF-1β, HIF-1α levels were also found to be down-regulated in type 2 diabetic islets. Also, beta-cell specific HIF-1α knock-out mice had impaired GSIS and were glucose intolerant, leading to hyperglycemia. In an attempt to understand whether the observed phenotype could be

rescued by improving HIF-1α levels, treatment with iron chelators, which has been shown to increase HIF-1α protein levels, resulted in improved insulin secretion and it normalized ARNT/HIF-1β mRNA and downstream target gene expression in type 2 diabetic islets. This is the firs study that has shown that increasing HIF-1α protein levels positively influences ARNT/HIF-1β mRNA levels. However, the exact mechanism of this interaction is still not completely understood.

1.11.2 ARNT/HIF-1β and Liver

In both rodents and humans, the liver plays a critical role in maintaining glucose and lipid homeostasis. During fasting, hepatic glucose production is critical in providing glucose for the brain, the kidneys and the red blood cells. In liver, glucose is produced by glycogenolysis during the initial stages of fasting. However, after several hours of fasting, glucose is primarily produced from gluconeogenesis, a process by which the liver produces glucose from precursors such as lactate and pyruvate, thus preventing the body from going into hypoglycemic shock (171,172). Similar to the observation made by Gunton and co-workers in human type 2 diabetic islets and consistent with it, Wang et al in 2009 reported that ARNT/HIF-1β was severely reduced in the livers of type 2 diabetic patients (173). Gene expression profiling of liver specimens from normal, obese and obese diabetic patients revealed a 30% reduction in the expression of ARNT/HIF-1β gene in obese diabetic individuals. The study demonstrated that the reduced expression of ARNT/HIF-1β in the livers of humans with T2D was associated with high glucose levels, high insulin levels and insulin resistance. Wang et al also demonstrated that insulin, not glucose, regulates the expression of the ARNT/HIF-1β gene in the liver and that its expression is reduced in both insulin-deficient and insulin-resistant states.

To understand the deleterious effects of disruption of the ARNT/HIF-1β gene in the liver, a liver specific ARNT/HIF-1β knockout mouse was generated (L-ARNT KO) (173). L-ARNT-KO mice exhibited increased gluconeogenesis, lipogenesis and serum insulin levels, characteristic of human T2D. The association between the loss of ARNT/HIF-1β and increased hepatic gluconeogenesis and lipogenesis may be mediated by increased expression

of several important gluconeogenic and lipogenic genes including PEPCK, G6Pase, SCD1 and FAS. Expression of C/EBPα and SREBP-1C, was also induced by 2-fold in L-ARNT KO mice. C/EBPα plays a major role in kick-starting hepatic glucose production at birth, and disruption of the C/EBPα gene in mice is known to cause hypoglycemia associated with the impaired expression of the gluconeogenic enzymes PEPCK and G6Pase (174,175). SREBP-1C, on the other hand, is a major player in lipogenesis (176). Thus, based on the above evidence, it seems that ARNT/HIF-1β acts as an upstream regulator of these transcription factors and plays a key role in maintaining whole body glucose and lipid homeostasis. However, as seen in pancreatic beta-cells, the exact pathways targeted by ARNT/HIF-1β in liver are not clearly understood and is complicated by the fact that ARNT/HIF-1β has multiple binding partners.

1.11.3 ARNT/HIF-1β and Adipose Tissue

Obesity leading to insulin resistance is one of the key pathophysiological abnormalities in T2D. Adipokines such as leptin and adiponectin secreted from the adipose tissue play a critical role in maintaining glucose metabolism, energy expenditure and inflammatory response (177,178). The occurrence of adipose tissue hypoxia is a characteristic feature of insulin-resistant obese state and exposure to hypoxia is known to have deleterious effects on adipose tissue. Wang et al reported that cultured adipocytes exposed to hypoxia showed increased expression of HIF-1α levels and this was associated with increased expression of inflammatory cytokines such as IL-6, raised leptin levels and decreased adiponectin levels (179). Similar observations were made in the adipose tissue of Ob/Ob mice and dietary obese mice, where hypoxia increased the expression of inflammatory genes and reduced the level of adiponectin, suggesting a potential role for hypoxia in the induction of chronic inflammation and inhibition of adiponectin in obese adipose tissue (180,181). Consistent with the deleterious effects of hypoxia and increased expression of HIF-1α protein, adipocyte-specific deletion of ARNT/HIF-1β in mice leads to protection of these mice from the consequences of high fat diet. Adipocyte-specific ARNT/HIF-1β knock-out mice were lean, had smaller adipocytes and were protected from age and diet-induced glucose

intolerance (182). These effects of ARNT/HIF-1β in adipose tissue were found to be mediated in part through HIF-1α. However, the effects HIF-1α deletion in adipocytes is inconclusive as studies conducted by two research groups yielded completely opposite results in mice. While Jiang et al's (183) observations were similar to those seen in adipocyte-specific ARNT/HIF-1β knock-out mice, where the adipocyte-specific HIF-1α mice were lean and protected from the effects of high fat diet, Zhang et al's study showed entirely opposite effects. Zhang and co-workers showed that adipocyte-specific deletion of HIF-1α leads to obesity and glucose intolerance by impeding energy expenditure (184). What makes this more complicated is the observation that a global knock-out of asparginyl hydroxylase, a factor inhibiting HIF-1α (FIH) function, leads to reduced body weight, elevated metabolic rate, hyperventilation and improved glucose and lipid homeostasis (185). The mice are also resistant to high-fat-diet-induced weight gain and hepatic steatosis suggesting that a global increase in HIF-1α levels is beneficial to mice. An interesting observation noted in their research was that the neuron-specific knock-out of FIH phenocopies some of the major metabolic effects observed in global FIH-knock-out mice suggesting that HIF signalling may play a critical role in the central control of metabolism. Thus, based on these results, although it is difficult to interpret the precise role of the HIF-1α-ARNT/HIF-1β partnership in the adipose tissue, it is quite evident that the role played by this heterodimeric duo is much more complex than in other tissues such as the liver and the beta-cells.

1.12 Objectives and Hypothesis

The main objective of this study was to understand the biochemical and physiological role of ARNT/HIF-1β in maintaining glucose-stimulated insulin secretion (GSIS) and glucose homeostasis in mice. In their study, Gunton and co-workers clearly demonstrated a role for ARNT/HIF-1β in maintaining normal beta-cell function, GSIS and glucose homeostasis in mice. However, a detailed analysis of the metabolic phenotype of beta-cells deficient in ARNT/HIF-1β and the mechanism by which the transcription factor regulated GSIS from pancreatic beta-cells was not fully investigated. Specifically, the extent of involvement of ARNT/HIF-1β in the regulation of key metabolic pathways such as the glucose metabolism pathway, which play a critical role in the control of GSIS, was largely unknown. The two specific objectives of the present study, therefore, are:

1. To determine the involvement of ARNT/HIF-1β in beta-cell glucose metabolism and insulin secretion using an siRNA against ARNT/HIF-1β in the 832/13 clonal beta-cell line
2. To determine the mechanism by which ARNT/HIF-1β maintains glucose homeostasis *in vivo:*
   - **(1)** Generate pancreatic beta-cell specific ARNT/HIF-1β knock-out mice (β-ARNT KO)
   - **(2)** Characterize *in vivo* metabolic parameters of β-ARNT KO mice
   - **(3)** Characterize *in vitro* beta-cell function of islets from β-ARNT KO mice

Hypothesis: Based on the known role of ARNT/HIF-1β as a protein required for the transcriptional activation of glycolytic enzymes in response to hypoxia, we hypothesize that ARNT/HIF-1β regulates GSIS by altering the key biochemical events in the glucose metabolism pathway. As a consequence of altering the biochemical events, we expect to see concomitant changes in the amounts of metabolites in the glucose metabolism pathway and their tributaries such as the amino acid and the fatty acid pathways, thereby regulating the metabolic signal transduction pathways that control GSIS from pancreatic beta-cells.

Chapter 2
Experimental Procedures

2.1 Methodology and Models

The functional significance of ARNT/HIF-1β in pancreatic beta-cells was investigated systematically through a loss of function strategy. RNA interference technology (RNAi), one of the most commonly used gene knock-down platforms, was used to transiently inhibit ARNT/HIF-1β mRNA and protein levels in the 832/13 clonal beta-cell line. In addition, the *in vivo* role of ARNT/HIF-1β was investigated through the disruption of the ARNT/HIF-1β gene specifically in the pancreatic beta-cells of mice using a widely used gene knock-out platform called the Cre-loxP recombination technology, the details of which are presented in section 2.14 of this chapter. The *in vivo* and *in vitro* characterization of beta-cells deficient in ARNT/HIF-1β was carried out using a number of biochemical assays and molecular biology techniques. The detailed experimental procedures used for performing these assays and techniques are described in the following sections.

2.2 832/13 Clonal Beta-Cell Line

832/13 cells were a kind gift from Dr. Christopher Newgard. These clonal cells were derived from rat insulinoma cell line, INS-1, by a transfection-selection strategy (186). Briefly, INS-1 cells were stably transfected with a plasmid containing the human proinsulin gene. After antibiotic selection and clonal expansion, only 16% of the clones were found to be strongly responsive to glucose in terms of insulin secretion and these were designated as 832/13 cells. These cells showed an average of 10-fold increase in insulin secretion when the glucose concentrations were raised from low to high. Along with a strong response to glucose, 832/13 cells were stable for 66 population doublings, which happened over 7.5 months, with a half-maximal stimulation at 6 mM glucose. Further characterization revealed that 832/13 cells responded strongly to all major insulin secretagogues and in some cases responses were stronger than the parental INS-1. For cell culture, 832/13 cells were cultured in RPMI 1640 medium containing 11.1 mM glucose (Fisher Hyclone - SH30027-FS) and supplemented with 10% FBS, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM β-

mercaptoethanol at 37˚ C in a humidified atmosphere containing 5% CO_2. Cells were maintained in a T75 flask at 1:3 ratio (cells: fresh culture medium) and passaged 2 - 3 times a week.

2.3 Silencing RNA (siRNA) Mediated Gene Suppression

Expression of ARNT/HIF-1β mRNA was silenced via introduction of 25-27 base pair siRNA duplexes (Integrated DNA Technologies (IDT), San Diego). Two siRNA duplexes were constructed against ARNT/HIF-1β mRNA (Accession number: NM_012780), siARNT 1 and siARNT 2. An siRNA sequence with no known gene homology was used as a control siRNA (siControl) for all experiments. Relative to the start codon, the 5'end of the siRNA target sequence corresponded to the following nucleotides in ARNT/HIF-1β as shown in table 1:

Table 1:List of siRNA sequences

Sequence Name	Start nucleotide	siRNA sequence (5'- 3')
siARNT 1	389	CCA UCU UAC GCA UGG CUG UUU CUC A
siARNT 2	891	GGA AGG AGA GCC UCA CUU UGU GGT A
siControl	NA	GAG ACC CUA UCC GUG AUU A

Transfection: 832/13 cells were plated at 0.5 million cells/mL/well of RPMI in a 12 well plate or 1 million cells/2 mL/well on a 6 well plate. Approximately 60 pmoles of siRNA duplexes were introduced per well of a 12 well plate at 50% confluence using Lipofectamine RNAiMax according to the manufacturer's instructions (Invitrogen). Cells were incubated with the transfection complex containing siRNA duplex and the lipofectamine RNAiMax for 24 h. Following 24 h transfection, transfection medium was changed and fresh RPMI growth medium was added and cells were cultured for an additional 48 h prior to metabolic assays, at which point cells were collected for RNA and protein.

2.4 RNA Isolation, cDNA synthesis and Real time PCR

RNA isolations from 832/13 cells or islets were carried out using an Aurum Total RNA Mini kit (BioRad, Hercules, CA). Approximately 0.5 μg of cDNA was synthesized through reverse transcription using an Iscript cDNA synthesis kit (BioRad, Hercules CA). Approximately 50 – 100 ng of cDNA were used for real time PCR analysis, which was performed on Applied Biosystems, ABI 7500 series real time PCR machine using appropriate primer sets (table 2) and SsoFast EvaGreen real time PCR supermix (BioRad) OR using pre-designed fluorescent probes purchased from Applied Biosystems, Inc. (ABI, Foster City, CA) (table 3). PCR primer sets were designed using Primer-BLAST, a primer-designing tool from NCBI and purchased from IDT (IDT, San Diego). Gene expression levels were corrected by cyclophilin E (Cyp) expression.

Table 2: List of PCR primers used for gene expression profiling

Gene	NCBI ID	Primer Sequence (Tm)	Product Size
ARNT	NM_012780.1	For: GCGGCGACGGAACAAGATGACA (62.2 ºC) Rev: ACACCACTCGGCCAGTCTCACA (62.7ºC)	266
CYP	NM_001047868.1	For: AGATGGCACAGGAGGAAAGAGCAT (60.3 ºC) Rev: AGGGTTTCTCCACTTCGATCTTGC (59.0 ºC)	302
CIC	NM_017307	For: TTGGGATGTTCGAGTTCCTCAGCA (60.3 ºC) Rev: TTGTCTCCTTGGTACCAGTTGCGT (60.5 ºC)	334
DIC	NM_133418	For: CCAGCAGATTTGGTCAATGTCAGG (58.3 ºC) Rev: GGTCCAAGCTTTGCTGTCTCCA (59.8 ºC)	392
OGC	NM_022398	For: TCTGTGCCACTTCTGCGCCA (62.1 ºC) Rev: AGCCACTGAGGAAGAGACGCT (60.4 ºC)	287
GK	NM_012565.1	For: ATGCTGGATGACAGAGCCAGGAT (60.4 ºC) Rev: TCGGGGATGGAGTACATCTGGTG (60.2 ºC)	335
GLUT 2	NM_012879.2	For: CCTGGCCGGGATGATTGGCA (62.9 ºC) Rev: AGGCCCGAGGAAGTCCGCAA (63.9 ºC)	283

Gene	NCBI ID	Primer Sequence (Tm)	Product Size
PC	NM_012744	For: TGACGTGGCCATGCGCTTCT (62.4 ºC) Rev: ACTGCCAGCTGCTTCCATGC (61.1 ºC)	250
PDH	NM_001004072	For: AGTCTGCTGCGCTCCATGAGG (62.8 ºC) Rev: CACACAGCAGGCTTCCTGACC (61.8 ºC)	321
FAS	NM_017332	For: TTTCCGCCATCTCCAAGACCTTCT (60.2 ºC) Rev: CAAGGTTCAGGGTGCCATTGTACT (59.3ºC)	390
CPT-1α	L07736	For: TGGCAGAGGCTCACCAAGCTGT (60.37 ºC) Rev: GGCTTGACATTCGGCCAGTGGT (59.67 ºC)	297
HNF-1α	NM_012669	For: TGGCTCAGCAATTCACCCACGC (60.24 ºC) Rev: T TGGCCAGCTTATGCCGAAAGGC (60.05 ºC)	330
HNF-4α	AF329936	For: CTCCAGTGGCGAGTCCTTATGA (59.8 ºC) Rev: AGCACTTCTTGAGCCTGCAGTA (58.6 ºC)	381
HIF-1α	NM_024359	For: AGCTCACCTGAGCCTAACAGTCC (60.5 ºC) Rev: TTGCGGTGGCAGTGACAGTGA (61.7 ºC)	322
PGC-1α	NM_031347.1	For: TGCCATTGTTAAGACCGA (61.2ºC) Rev: CCAGTAAACCACTGAGACC (61ºC)	300
EPAC 2	XM_215985.5	For: ATACTGCCAGAACGGTGCGCT (61.1 ºC) Rev: AAGCTACGGCATGTCCGCGTT (62 ºC)	377
ARNT Floxed	N/A	For: TGCCAACATGTGCCACCATG Rev: GTGAGGCAGATTTCTTCCATGCTC	WT (290) +/Fl (290, 340) Fl/Fl (340)
Cre	N/A	For: AAAATTTGCCTGCATTACCG (62ºC) Rev: ATTCTCCCACCGTCAGTACG (62ºC)	553
ARNT	NM_001037737.2	For: AGCGGTTTGCCAGGTCGGATG (59ºC) Rev: GATGTGTTGCCAGTTCCCCTCAA (58.2ºC)	232
PC	NM_001162946.1	For: GCTGTGCCATTCAGTGTCGGGT (59ºC) Rev: TGGGGTGGTCTTTGCCGTGT (60ºC)	201

Table 3: List of fluorescent probes used for gene expression profiling

Fluorescent Probes	NCBI ID	Reference ID	Product Size
MEc	NM_012600.2	Rn01445345_m1	112
ICDc	NM_031510.1	Rn01438266_m1	121
ACS	NM_023104.1	Rn01646009_g1	80
PLC-g1	NM_013187.1	Rn01640806_m1	114
CL	NM_001111095.1	Rn00566411_m1	89

2.5 Protein Isolation and Western Blot

Cellular proteins were extracted with cell lysis buffer (Cell Signalling, Danvers, MA) containing phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (10 µg/ml), aprotinin (10 µg/ml), and pepstatin (5 µg/ml). Protein quantification was performed using the bicinchoninic acid (BCA) protein assay. In the BCA assay, total protein extracted with cell lysis buffer was compared to protein standards through a colorimetric assay. The assay exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. Protein extracts (40 µg) were resolved on 10% Bis-Tris SDS-polyacrylamide gels and electro transferred to nitrocellulose membranes (Invitrogen). ARNT/HIF-1β was detected with a monoclonal antibody against ARNT (1:1000) (BD Bioscience) followed by horse-radish peroxidase-conjugated anti-mouse antibody (1:10,000) (Amersham Biosciences). γ-Tubulin was detected by immunoblotting with a mouse antibody against γ-tubulin (1:4000) (Sigma) followed by horseradish peroxidase-conjugated antimouse antibody (1:15,000) (Amersham Biosciences). Protein bands were detected with the ECL Advance immunoblot detection kit (Amersham Biosciences). All protein bands were visualized and quantified using a Kodak Imaging ChemStation (187).

2.6 Glucose-Stimulated Insulin Secretion Assay – 832/13 Cell line

832/13 cells were plated on either a 6 well plate with an initial seeding density of 1 million cells/2mL/well or a 12 well plate with an initial seeding density of 0.5 million cells/mL/well and cultured for 5 days in RPMI 1640 medium with all nutritional supplements as noted in section 2.1. Insulin secretion was measured by pre-incubating cells in Kreb's ringer buffer (KRB) containing 129 mM NaCl, 4.7 mM KCl, 1.5 mM KH_2PO_4, 1.16 mM $MgSO_4$, 10 mM HEPES, 5 mM $NaHCO_3$, 3.11 mM $CaCl_2$, 0.2% bovine serum albumin, pH 7.4 with 2 mM glucose for 2 x 1h at 37^o C with 5% CO_2. Following 2 x 1h pre-incubation, cells were incubated in KRB containing either 2 mM, 7 mM or 16.7 mM glucose as indicated for 2 h at 37^o C with 5% CO_2. At the end of the 2 h incubation, the plates were gently swirled and samples (500 μL) of KRB from each well were removed and stored in appropriately labeled eppendorf tubes until insulin was measured through a radioimmunoassay (Coat a Count, Siemens) according to the manufacturer's directions. All insulin values were normalized to protein content (186).

For treatment with various insulin secretagogues, secretagogues were directly added to 2 mM, 7 mM or 12 mM glucose as indicated on the day of the assay. 3 M KCl stock was prepared in water and diluted to 30 mM in freshly prepared KRB. Diazoxide was prepared in 0.05 M NaOH stock solution stored in -20 ^{o} C and was diluted in freshly made KRB to reach the final concentration (100 μM in 832/13 cells and 200 μM in mice islets). For the preparation of amino acids, both leucine and glutamine were added to freshly prepared KRB (weight/volume) without glucose to reach a final concentration of 10 mM each in solution. For low amino acid concentration, 10 mM solution was diluted 1:10 in KRB without glucose. For dimethyl malate (DMM – Sigma 374318) and dimethyl alpha ketoglutarate (DMAKG – Sigma S84045), 5 mM each of the stock solution (density/volume) was added to freshly prepared KRB solution on the day of the assay.

2.7 Preparation of Palmitate complex for GSIS

1 mM Palmitate /0.17 mM BSA – Caution: 1) Use glass vials for preparation of palmitate-BSA as the complex will readily adhere to plastic. 2) Maintaining suggested temperatures for preparation of the complex is critical to achieve quality conjugation.

A stock of 2 mM palmitate solution was prepared by dissolving sodium palmitate (Sigma P9767) in 50 mL of 150 mM NaCl at 70 ^{o} C. To minimize vapour loss, the beaker was covered with parafilm and NaCl/Palmitate solution was heated to 70°C on a hot plate, with continued stirring and monitoring of the temperature using a thermometer. 2 % Fatty Acid-Free (FFA) BSA solution was prepared in 100 mL KRB (without the phosphates – Islet KRB) at 37 **^{o}** C and filtered using 0.45-micron filter. Half of the BSA solution was diluted with phosphate-free KRB to get a 1% BSA solution and stored at -20 **^{o}** C**.** This was used as a vehicle control. The remaining half of the BSA solution was used for the preparation of palmitate-BSA complex.

Once the palmitate and BSA solutions were ready, 40 mL of the palmitate solution at 70 **^{o}** C was slowly transferred to the BSA solution at 37 **^{o}** C while stirring. Since palmitate solution will precipitate if it is allowed to sit in the pipette, 5 mL of the palmitate was transferred at a time to the BSA solution, aspirating and dispensing quickly. Once the transfer was done, the beaker was covered with parafilm while continuing to stir at 37 ^{o} C for 1 h, monitoring the temperature of the water bath to stay between 37 ^{o} C and 40 ^{o} C. Once the palmitate was completely dissolved in BSA solution, pH was adjusted to 7.4 and a final volume to 100 mL with phosphate-free KRB solution. The palmitate-BSA complex was filtered using 0.45-micron filter and stored in glass vials at -20 ^{o} C. For use in INS-1 derived 832/13 cells, since the palmitate-BSA complex is prepared in phosphate-free KRB, phosphates were added in appropriate amounts at a later stage when the complex preparation was completed and before storing the solution at -20 **^{o}** C**.** The amount of free-fatty acids in the solution was measured using a NEFA kit (HR Series NEFA-HR (2), Wako Diagnostics) and the concentration of palmitate needed for GSIS was achieved by diluting with KRB (188).

2.8 Glucose Utilization

832/13 cells were plated on a 12 well plate at an initial seeding density of 0.5 million cells/mL/well. Cells were transfected with siRNA duplexes and cultured for a total of 72 h post transfection. On the day of the assay, cells were pre-incubated in KRB containing 2 mM glucose for 2 h, followed by 2 h treatment in KRB containing either 2 mM or 16.7 mM [5-^{3}H] glucose (Perkin Elmer) as a tracer at a specific activity of 0.08 Ci/mol. At the end of 2 h treatment with tracer, KRB was collected and protein was precipitated using 10% TCA. Following centrifugation at 12,000 x g for 5 min, the remaining buffer was transferred to a capless eppendorf tube placed inside a scintillation vial, and allowed to equilibrate overnight at 50°C in a CO_2 -free incubator. Following overnight incubation, vials were allowed to cool to room temperature for 1 h, capless tubes were removed and the vials were counted using liquid scintillation fluid (Economical Biodegradable Counting Cocktail; Econosafe, Research Products International (RPI), Mount Prospect, Illinois). Efficiency of the equilibration was determined by measuring equilibration of $^{3}H_2O$ (Amersham). All glucose utilization values were normalized to protein content (189).

2.9 Glucose Oxidation

832/13 cells were plated on a 12 well plate at an initial seeding density of 0.5 million cells/mL/well. Cells were transfected with siRNA duplexes and cultured for a total of 72 h post transfection. On the day of the assay, cells were pre-incubated in KRB containing 2 mM glucose for 2 h, followed by 2 h treatment in KRB containing either 2 mM or 16.7 mM [U-^{14}C] glucose (Perkin Elmer) as a tracer at a specific activity of 0.5 Ci/mol. Before 2 h incubation of cells with tracer, each well containing cells was loaded with a trap system containing 1 N NaOH. The trap system was tightly sealed and incubated at 37 ^{o} C for 2 h. At the end of 2 h incubation, 70% perchloric acid was carefully injected into each well for protein precipitation. The trap was then incubated on a shaker at 125 rpm for 90 min. NaOH was transferred to scintillation vials containing UniScint BD scintillation fluid (BD Biosciences), mixed, and then counted to determine glucose oxidation rates and normalized to protein content (74,189).

2.10 ATP – ADP Measurements

832/13 cells were plated on a 6 well plate at an initial seeding density of 1 million cells/mL/well. Cells were transfected with siRNA duplexes and cultured for a total of 72 h post transfection. On the day of the assay, cells were pre-incubated in KRB containing 2 mM glucose for 2 h, followed by 2 h treatment in KRB containing either 2 mM or 16.7 mM glucose. At the end of the 2 h treatment, KRB buffer was collected for insulin assay. Cells were scraped off and snap frozen in a dry-ice ethanol bath and stored at -80 ^{o}C until the day of the ATP-ADP assay. ATP and ADP were extracted from the samples with 20 units of 10% (w/v) perchloric acid. After centrifugation, the supernatants were neutralized with 2 N KOH and 0.5 M triethanolamine. Aliquots of each deproteinized sample were assayed for ATP directly with luciferase. Other aliquots were treated with ATP sulfurylase in the presence of molybdate to hydrolyze the endogenous ATP to AMP and $PP_{i.}$ After boiling the samples to inactivate the sulfurylase, ADP was then converted to ATP with pyruvate kinase. ATP content in these samples was measured with luciferase (Sigma L9506) in the presence of luciferin (Sigma L9504) mixed with ATP assay mix dilution buffer (Sigma- FLAAB-1VL) Luminescence was measured using a spectrophotometer and normalized to protein content (190).

2.11 NADPH-NADP Measurement

832/13 cells were plated on a 6 well plate at an initial seeding density of 1 million cells/2mL/well. Cells were treated with siARNT or siControl for 72 h. On the day of the assay, cells were pre-incubated in KRB containing 2 mM glucose for 2 h, followed by 2 h treatment in KRB containing either 2 mM or 16.7 mM glucose. At the end of the 2 h treatment, KRB buffer was collected for insulin assay. Cells were scraped off and snap frozen in dry-ice ethanol bath and stored at -80 ^{o}C until the day of the NADPH-NADP assay. The assay was performed using a NADPH/NADP quantification kit from Sigma (MAK038) following the manufacturer's instructions. The assay is specific for NADP and NADPH and does not detect NAD or NADH. NADP (total) (NADP and NADPH) and NADPH are quantified in a colorimetric assay (450 nm) (67).

2.12 Gas Chromatography- Mass Spectrometry Analysis of Metabolites (GC-MS

832/13 cells were plated on a 6 well plate at an initial seeding density of 1 million cells/2mL/well. Cells were treated with siARNT or siControl for 72 h. On the day of the experiment, cells were first incubated for 2 h at low glucose (2.8 mM) followed by incubating the cells with either low (2.8 mM) or high glucose (16.7 mM) for 2 h. After the insulin secretion assay, cells were washed once with ice-cold PBS, scraped off the plates followed by centrifugation at 3,500 rpm for 1 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 100 µl of water followed by sonicating for 60 sec (~40 KHz, ~140 W). One ml of methanol was added followed by centrifugation for 5 min at 13,300 rpm. Supernatant was mixed with 5 µl of 0.25 mg/ml myristic acid-d27 as an internal standard (used for retention time locking) followed by drying with nitrogen. Derivatization was then performed in two steps: first, carbonyls were protected by methoximation using methoxyamine hydrochloride in pyridine at 50°C for 30 min. Second, acidic groups were silylated with 90 µl N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% TMCS at 50°C for 30 min.

GC-MS analysis: One microliter of the derivatized sample was injected in split mode with 1:2 split ratio onto a GC (7890A Agilent, USA; DB-5MS column (30 min × 250 µm × 0.25 µm)). Detection was performed with a quadrupole mass spectrometer (5895C Agilent USA). Each sample was run four times.

Deconvolution and Identification: The Automated Mass Spectral Deconvolution and Identification System software (AMDIS, Version 2.65) was used for peak detection and mass spectrum deconvolution. Target compounds were identified using retention time and by matching mass spectra in either Fiehn GC/MS Metabolomics Retention Time Locking (RTL) Library (Version 2.0f) or NIST library (2008). A known amount internal standard (myristic acid-d27) was used to calculate relative metabolite levels **(191)**

2.13 Animal housing

All mice were housed in the temperature controlled central animal facility of the University of Waterloo and were handled according to the Canadian Council of Animal Care guidelines. All animal studies were approved by the Office of Research Ethics, University of Waterloo-(Animal Utilization Project Proposal – AUPP 11-19). Mice were subjected to a 12 h light and dark cycle and were fed on breeder chow. All mice used in this study were from a C57BL/6 genetic background.

2.14 Generation of ARNT/HIF-1β Knock-Out Mice

The generation of pancreatic beta-cell specific ARNT/HIF-1β knock-out mice (β-ARNT KO) requires two types of transgenic mice: the ARNT Floxed mice ($ARNT^{Fl/Fl}$) and mice expressing Cre-recombinase under the influence of rat insulin promoter (RIP-Cre). Briefly, tissue specific expression of Cre-recombinase in pancreatic beta-cells is achieved by keeping the Cre under the control of rat insulin promoter (RIP). When pancreatic beta-cells that have *loxP* sites in their genome express Cre-recombinase, the Cre protein will recognize and cut the *loxP* sites, leading to deletion of the floxed target DNA sequence. Floxed mice were generated by integrating the *loxP* sites on either side of exon 6 of the ARNT gene (163). The RIP-Cre transgene was constructed by the ligation of 668 nucleotides of the rat insulin promoter sequence to the coding sequence of Cre with a nuclear localization signal and a poly adenylation signal (192). Since ARNT floxed allele has the *loxP* sites flanking exon 6 of the ARNT gene, Cre-mediated recombination event will lead to the deletion of exon 6, resulting in the loss of ARNT protein function (Figure 6).

Homozygous ARNT Floxed mice were a kind gift from Frank Gonzalez (NIH) and RIP-Cre mice were a kind gift from Dr. Mike Wheeler (University of Toronto). ARNT floxed mice and RIP-Cre mice were bred together to generate the knock-out mice at the Central Animal Facility, University of Waterloo.

β-ARNT KO mice were generated in a two-step breeding process: In the first step, mice expressing the RIP-Cre transgene were bred to homozygous ARNT Floxed mice ($ARNT^{Fl/Fl}$), resulting in the generation of mice heterozygous for Floxed ARNT ($ARNT^{WT/Fl}$) and

containing a copy of the RIP-Cre transgene (F1). In the second set of breeding, the F1 heterozygotes were bred together to generate F2 mice, one fourth of which were homozygous for the ARNT Floxed gene with a copy of the Cre transgene ($ARNT^{Fl/Fl}$,Cre). The presence of RIP will ensure that Cre-recombinase is expressed only in the beta-cells resulting in the tissue specific deletion of ARNT protein (193). Mice were born in the expected Mendelian ratio and the knock-out mice were phenotypically similar to their control littermates.

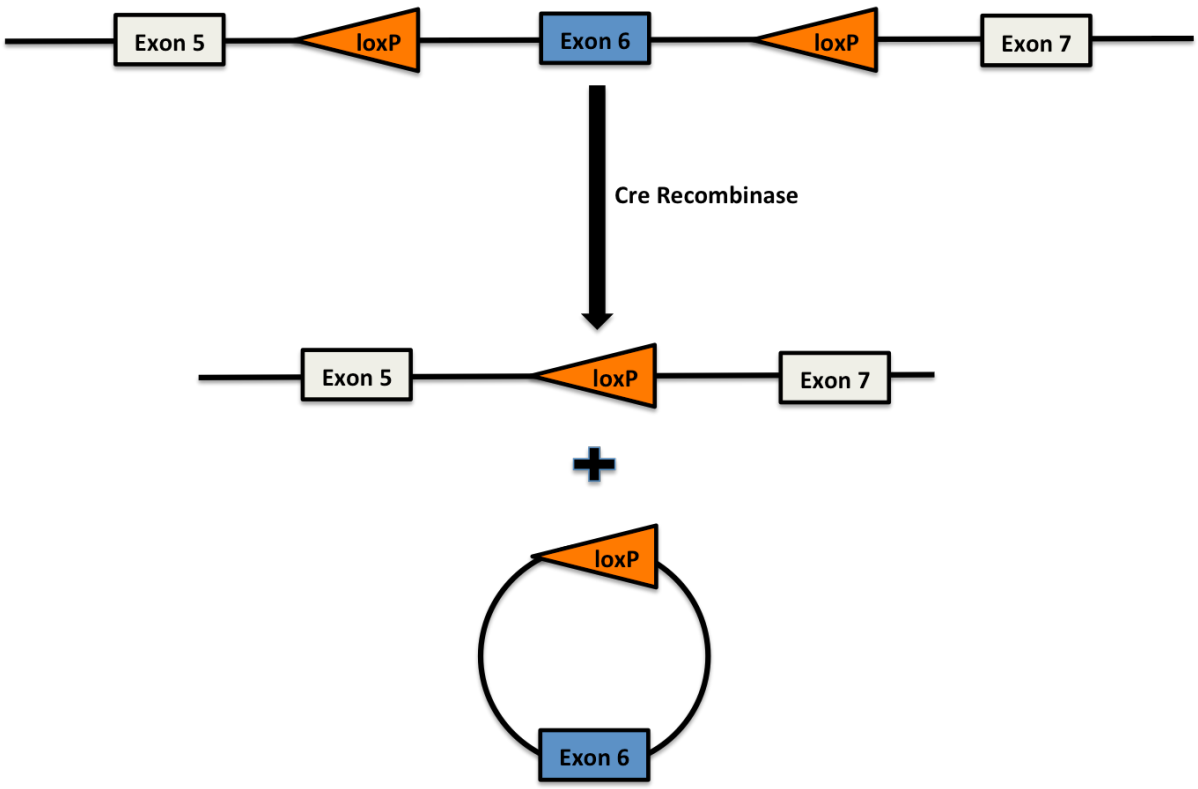

Figure 6: Schematics of Cre mediated DNA recombination. The Cre protein, which is a site-specific DNA recombinase, catalyzes the recombination of DNA between the *loxP* sites flanking exon 6 of ARNT gene resulting in the deletion of exon 6 that encodes the bHLH region of the ARNT protein. Briefly, the double stranded DNA is cut at both *loxP* sites and the strands are then rejoined by DNA ligase. The deletion process also results in the formation of a covalently closed circular DNA.

2.15 Genotyping – DNA Extraction and PCR

Mice were ear-notched and DNA was extracted from the ear-clips using PureLink™ Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instructions. Briefly, ear notches were digested using genomic digestion buffer and proteinase K for 3 - 4 hr at 55 ^{o} C in a heating block. Residual RNA was removed by RNase digestion prior to sample binding to the silica membrane. The lysate was mixed with ethanol and PureLink™ genomic binding buffer that allows high DNA binding to the silica-based membrane in the column. Impurities were removed by thorough washing with Wash Buffers 1 and 2. The genomic DNA was then eluted in low salt elution buffer.

PCR was carried out to determine the presence of WT (290 bp), ARNT $^{WT/Fl}$ (290 and 340 bp), ARNT$^{Fl/Fl}$ (340 bp) and RIP-Cre (553 bp) transgenes using a Platinum Blue PCR supermix (Invitrogen) according to the manufacturer's instructions. The following thermal-cycling profile was used for the detection of the floxed ARNT gene: 1 cycle of 5 min at 95°C, 30 cycles at 95°C for 45sec, 60°C for 45 sec, 72°C for 45 sec and a final extension of 1 cycle of 72°C for 6 min. PCR to detect the RIP-Cre transgene was achieved with 35 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. PCR products were separated on 1% agarose gels using 0.5 X TAE (20 mM Tris-acetate and 2 mM $Na_2EDTA.2H_2O$, pH 8.5) as the running buffer and were visualized under UV light after staining with 0.1% ethidium bromide. A 100 bp low-scale DNA ladder was used for size comparison (Fischer – B2581-200). A list of PCR primers used for genotyping is given in table 2.

2.16 Intraperitoneal Glucose Tolerance Test (ipGTT)

Age matched (15-20 weeks) male and female mice were fasted for 16 h, weighed and the tails were nicked with a fresh razor blade and gently massaged to collect 5 -10 μL of blood sample on to a glucose test strip. Basal glucose measurements were taken (0 min) followed by an intraperitoneal injection of glucose (1.5g/kg). Glucose solution was freshly prepared for each experiment in sterile phosphate buffered saline (PBS). Blood glucose measurements were taken at 10, 20, 30, 60 and 120 minutes using a Bayer Contour blood glucose monitoring system (Bayer Healthcare, Canada)

2.17 Insulin Tolerance Test (ITT)

Age matched (15-20 weeks) male and female mice were fasted for 4 h, weighed and the tails were nicked with a fresh razor blade and gently massaged to collect 5 -10 μL of blood sample on to the glucose test strips. Basal glucose measurements were taken (0min) followed by an intraperitoneal injection of exogenous insulin (Humulin R- Lilly, Canada. 1.2 U/Kg body weight for females and 1.5 U/Kg body weight for males). Stock insulin (100 U/mL) was diluted in sterile PBS. Blood glucose measurements were taken at 10, 20, 30, 60 and 120 minutes using a Bayer Contour blood glucose monitoring system (Bayer Healthcare, Canada).

2.18 Comprehensive Lab Animal Monitoring System (CLAMS)– Assessing metabolic phenotype through indirect calorimetry

Using indirect calorimetry, we examined the following parameters: VO_2, VCO_2, respiratory quotient (RER-VCO_2/VO_2), heat production, food intake and dual beam activity. All parameters were normalized to body weight. Male mice between15-20 weeks of age were used for this study. CLAMS with O_2 and CO_2 sensors and an infrared system to monitor X,Y,Z activity was used (Oxymax, Columbus Instruments, Columbus, OH). Both control and knock-out animals were studied simultaneously in individual chambers connected to the same O_2 and CO_2 sensors in an effort to minimize the effect of environmental variations and calibration on data. After a 24 h acclimatization period, mice were monitored in the metabolic chambers for an additional 24 h with ad-libitum access to food and water. Gas samples were collected and analyzed every 30 minutes per animal and the data collection was performed by an integrated program and exported to an EXCEL file. Output parameters include VO_2 (mL/kg/min), VCO_2 (mL/kg/min), heat production (calories/h) RQ (VCO_2/VO_2), food intake (grams/24 h) and dual beam activity. All measurements were averaged over each 30 minutes of the 24-h period from each group (n = 8 per genotype). An average of two separate trials were used for data analysis (194).

2.19 Islet Isolation

Mice were anesthetized by intraperitoneal injection of approximately 1μL/g of body weight

of sodium pentabarbitol (NaP). (NOTE: NaP is a controlled substance and must be stored in a locked cabinet). Once the mice are anesthetized, they were surgically opened and the pancreas was perfused through the common bile duct with about 3 - 4 mL of liberase (0.1WU/mL, Liberase TL Research Grade, Roche) made in 1 x Hank's buffer (136 mM NaCl, 0.3 mM NaH_2PO_4, 4.16 mM $NaHCO_3$, 5.36 mM KCl, 0.4 mM KH_2PO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES). The perfused pancreas was then extracted from surrounding tissues and digested at 37 °C for approximately 10 min. Digestion time is dependent on the enzyme activity and perfusion status of the pancreas. Once the digestion is completed, ice-cold Hanks'media was added immediately to arrest the digestion process. Islets were then sieved and washed in 2 x basic medium (RPMI 1640 with 1% HEPES) by spinning them at 1000 rpm for 30 sec and removing the supernatant with each wash carefully without losing the pellet. The pellet containing the islets was then resuspended in islet culture medium (RPMI 1640 with 1.25% FBS, 1% HEPES, 0.1 % glutamine, 0.02 % of penicillin/streptomycin). Islets were then hand picked using a dissection microscope and cultured overnight in islet culture medium in a 6 well plate with approximately 200 islets/ 5 mL /well and at 37^o C with 5% CO_2 (88).

2.20 Glucose-Stimulated Insulin Secretion - Islets

For the islet secretion assay, islets were washed and incubated for 2 x 30 min in 5 mL of KRB containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 5 mM HEPES, 2.5 mM $CaCl_2$, 24 mM $NaHCO_3$, 0.1% bovine serum albumin, pH 7.4, with 2 mM glucose in a 6 well plate with approximately 100 islets/well at 37^o C with 5% CO_2. Islets were then divided into groups of 10 in a 1.5 mL eppendorf tube with 500 µl KRB buffer containing either 2 mM or 16.7 mM glucose for 1 h at 37^o C with 5% CO_2. After 1 h incubation, eppendorf tubes were taken out of the incubator and KRB buffer was gently mixed and left at room temperature for 5 min, allowing the islets to settle at the bottom. 400 µl of KRB was aspirated from each tube and stored at -20 ^{o} C until the day of the radio immuno assay (RIA) for insulin. RIA for measuring insulin in samples was carried out using Coat A Count Kit (Siemens) according to manufacturer's directions (88).

2.21 Mitochondrial Membrane Potential

Mitochondrial membrane potential was quantified in whole islets using a mitochondrial-specific dye, rhodamine 123. Islets were loaded with rhodamine 123 (25 ug/ml, 10 min) in Krebs-Ringer buffer (KRB) containing 1 mM glucose, 0.1% BSA, 120mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM $NaHCO_3$, and 10 mM Hepes (pH 7.3) and fluorescence was measured for 100 secs at 37^o C. After 100 sec at basal glucose concentration and achieving a stable baseline, 20 mM glucose was added for the next 400 sec. At the end of the experiment, 1 mM sodium azide (NaN_3) was added to inhibit the respiratory reaction and fully dissipate the potential. Membrane potential was measured using an Olympus IX70 inverted epifluorescence microscope connected to an Ultrapix camera and a computer with Merlin imaging software (LSR) (195).

2.22 Cellular Oxygen Consumption Measurement

Cellular oxygen consumption rates (OCR) were measured using a Seahorse flux analyzer (Seahorse Bioscience, Billerica, MA, USA). 40-50 islets were placed in XF24 islet plates following the protocol from Seahorse Biosciences and cultured overnight in islet culture medium (Section 2.19) at 37°C with 5% CO_2. This allows the islets to adhere to the bottom of the plate overnight. Following overnight culture, islets were gently washed in KRB (islet KRB – section 2.20) containing 2 mM glucose to remove any traces of the culture medium. After the washes, islets were incubated 2 x 30 minutes in KRB containing 2 mM glucose (0.5 mL) at 37°C with 5% CO_2. Following the incubation, KRB containing 2 mM glucose was gently aspirated and fresh 0.5 mL KRB with 2 mM glucose was added. At this point, the XF24 plate was ready to be loaded into XF24 Seahorse Flux Analyzer (Seahorse Bioscience) for OCR measurements. For 832/13 cells, cells were seeded at 50,000 cells per well in XF24 plates (Seahorse Bioscience) and treated with either siControl or siARNT using lipofectamine RNAimax as described before in section 2.3. On day 5, cells were pre-incubated in KRB buffer containing 2 mM glucose (0.5 mL) for 2 h, changing the solution after 1 h, adding fresh KRB and placed at 37°C with 5% CO_2. At this point plates are ready to be loaded into XF24 Seahorse Flux Analyzer (Seahorse Bioscience) for OCR measurements.

The cartridge (Seahorse Bioscience) was loaded with respective mitochondrial drugs prepared ahead of time and the loaded cartridge was kept at 37°C until the sample plate (XF24 islet or cell plate) was ready to be loaded to the Flux Analyzer. OCR was measured in response to 2 mM (basal) and 16.7 mM glucose, 5 μM oligomycin, 50 μM 2,4-dinitrophenol (DNP) and 10 μM rotenone + myxothiazol in the following configuration: mixing 2 min, waiting 2 min, measuring 3 min, loop x4. Raw OCR data was normalized to basal OCR. These values were then expressed as a percentage of the basal OCR. Normalized OCR values were then used to calculate different mitochondrial parameters such as ATP turn over, proton leak and spare respiratory capacity (196).

Mitochondrial drugs: The XF24 Seahorse Flux Analyzer is used to measure the bioenergetic profile of the mitochondria and therefore commonly used to study the oxygen consumption by the cells (197). The functional state of mitochondria is studied through the use of various chemicals or drugs that have been proven to interfere with the normal oxygen consumption processes in the mitochondria. Each drug generates a typical OCR profile specific for the cell-type used. Following OCR measurements under basal (2 mM) and high glucose (16.7 mM) conditions, the mitochondrial drug oligomycin is added. Oligomycin inhibits ATP synthase (198) and is used to isolate the portion of oxygen consumed that is used by proton leak. Next, an uncoupling drug, 2,4-dinitrophenol (DNP), was administered along with a fuel to ramp up the OCR to the maximum level possible. Uncouplers destroy the proton gradient and allow the protons to easily flow back across the mitochondrial membrane. This causes an increased flux of electrons through the electron transport chain as the mitochondria try to maintain the proton gradient. Thus, under the influence of an uncoupler, the maximum possible OCR is found. Finally, a combination of rotenone and myxothiazol were administered which completely inhibits mitochondrial respiration by the inhibiting the enzyme complexes of the electron transport chain and OCR readings observed were an indication of oxygen consumption from non-mitochondrial components of the cell.

2.23 Intracellular Calcium Measurement

Changes in intracellular calcium concentrations were assessed using dispersed islets plated on glass coverslips and cultured in islet culture medium with 5% CO_2 at 37°C. 24 – 36 h post dispersion, cells were first gently washed in islet KRB with 2 mM glucose and loaded with 2 μmol/l Fura-2 AM dye (Molecular Probes, Invitrogen) and incubated in a CO_2 incubator at 37°C for 50 min. At the end of the incubation, coverslips containing dispersed islets were loaded on the microscope and cells were perfused with KRB containing 2 mM glucose for 100 Sec, 20 mM glucose for 400 sec and with 20 mM glucose and 30 mM KCl for 200 sec at a flow rate of 1 mL/min at 37°C. Experiments were performed using an Olympus BX51W1 microscope (Tokyo, Japan) with an x20/0.95 water immersion objective and cooled charge-coupled device camera. Measurements were taken using ImageMaster version 3.0 software (Photon Technology International, London, ON, Canada). Analysis was performed using Igor Pro version 4.0 software (Wavemetrics, Lake Oswego, OR, USA) and normalized to baseline. Only cells showing a response to 30 mmol/l KCl solution were included for data analysis (199).

2.24 Statistical Test

Statistical significance was assessed by Student's t-test or analysis of variance post hoc Tukey's test using GraphPad Prism 4. A p-value of less than 0.05 was considered significant. All data is expressed as mean ± SEM.

Chapter 3

ARNT/HIF-1β Plays a Critical Role in Maintaining Glucose-stimulated Anaplerosis and Insulin Release from Pancreatic Beta-Cells

3.1 Synopsis

Despite its relevance in type 2 diabetes, the fundamental biochemical mechanisms and the metabolic pathways that regulate glucose-stimulated insulin secretion (GSIS) from pancreatic beta-cells are not completely understood. One potential regulator of the metabolic phenotype of beta-cells is the transcription factor ARNT/HIF-1β, which was found to be profoundly reduced in islets from type 2 diabetic patients. The main objective of this study was to investigate the biochemical mechanisms by which ARNT/HIF-1β regulates GSIS and identify the key metabolic pathways that are targeted by the transcription factor in the regulation of GSIS from pancreatic beta-cells. Here we confirm that siRNA-mediated knockdown of ARNT/HIF-1β inhibits GSIS in the INS-1 derived 832/13 cell line. We demonstrate that beta-cells with reduced ARNT/HIF-1β expression levels exhibit a 31% reduction in glycolytic flux without generating significant changes in glucose oxidation or the ATP/ADP ratio, suggesting that ARNT/HIF-1β mediates GSIS independent of rise in ATP/ADP ratio which is one of the most critical signals required for triggering GSIS. Metabolic profiling of 832/13 cells treated with siRNA against ARNT/HIF-1β reveals a significant reduction in glucose-induced rise in the amounts of metabolites generated in glycolysis, TCA cycle and fatty acid pathway. These changes in metabolite levels were associated with a corresponding change in the gene expression pattern of key enzymes regulating these biochemical pathways. Together, our data suggest that ARNT/HIF-1β is required to maintain glucose-stimulated anaplerosis and thereby regulates insulin secretion from the pancreatic beta-cells in a manner that is independent of rise in ATP/ADP ratio.

3.2 Introduction

Pancreatic beta-cells secrete the hormone insulin in response to a rise in the blood glucose levels. Abnormal insulin secretion is one of the earliest detectable defects at the onset of type 2 diabetes (28) and despite its relevance, the mechanisms underlying glucose-stimulated insulin secretion (GSIS) are not completely understood. It is therefore critical that we understand the exact mechanism of insulin release from pancreatic beta-cells to develop successful strategies in the treatment of type 2 diabetes.

The ability of the pancreatic beta-cells to maintain glucose homeostasis mainly depends on its capacity to secrete insulin in response to a change in arterial blood glucose concentration. It is generally accepted that the metabolism of glucose generates signals that are necessary for the acute stimulation of insulin secretion. The consensus model of GSIS holds that metabolism of glucose leads to an elevation of the ATP/ADP ratio to a point where it promotes the closure of the K_{ATP} channels, resulting in the depolarization of the beta-cell plasma membrane and opening of voltage-gated calcium channels. This allows calcium to enter the cytosol and promote insulin granule exocytosis (54,57,200). This so-called K_{ATP} channel-dependent pathway appears to be particularly important for the first, acute phase of insulin release. However, in the second and more sustained phase of insulin secretion, in addition to the initiation by the K_{ATP} channel dependent pathway, the K_{ATP} channel-independent mechanism seems to be necessary for the amplification of GSIS.

Important support for the K_{ATP} channel-independent pathways of GSIS comes from studies showing that glucose still causes a significant increase in insulin secretion in conditions where K_{ATP} channels are held open by application of diazoxide and high K^+, or in animals lacking functional K_{ATP} channels (58,79,201,202). Other studies have suggested that mitochondrial metabolism of glucose generates signals other than changes in the ATP/ADP ratio that are important for normal insulin secretion. Several molecules, including glutamate, malonyl-CoA/LC-CoA and NADPH, have been proposed as candidate coupling factors in GSIS (65,66,68,69,71,203,204).

Transcription factors regulate a variety of pancreatic beta-cell processes such as cell differentiation, proliferation, cell signalling and apoptosis (205-209). Maintenance of

functional and mature beta-cell phenotype requires the optimal expression of key transcription factors. In the context of T2D, it is a well-known fact that abnormal gene expression contributes to a myriad of beta-cell abnormalities. Support for this comes from studies on maturity-onset diabetes of the young (MODY), a monogenic form of T2D characterized by an early onset of the disease and defects in insulin secretion leading to hyperglycemia. With the exception of MODY-2, which is caused by a mutation in glycolytic enzyme glucokinase (GK), MODY-1, 3, 4, 5 and 6 result from mutations in genes encoding transcription factors, hepatocyte nuclear factor (HNF) HNF-4α, HNF-1α, HNF-1β, Pdx-1, and NeuroD/BETA-2 respectively (210-217).

Among the large number of transcription factors identified as essential for the proper maintenance of beta-cell function, one of the recently emerged key players is the transcription factor ARNT/ HIF-1β. In an attempt to look at the expression profile of genes in human islets under normal and diabetic conditions, Gunton et. al discovered that ARNT/HIF-1β was the most down-regulated gene in type 2 diabetic islets and the expression was 90% less in diabetic human islets (164). The importance of ARNT/HIF-1β in GSIS was further confirmed by the diminished GSIS in islets obtained from beta-cell specific ARNT/HIF-1β knock-out mice (β-ARNT KO) as well as in Min6 cells where the transcription factor was effectively silenced by siRNA technology. Furthermore, β-ARNT KO mice were glucose intolerant and islets from β-ARNT KO mice showed a significant decrease in the expression of genes that are critical for beta-cell secretory competence such as HNF-4α, the insulin receptor, IRS-2 and Akt2 which were all shown to be critical for maintaining glucose homeostasis. Several enzymes in the proximal part of the glycolysis pathway, including phosphoglucomutase (PGM), glucose-6-phosphate isomerase (G6PI), phospho fructokinase (PFK) and aldolase A (ALDO) were also found to be expressed at significantly lower levels in islets from β-ARNT KO mice compared to those observed in normal mice islets. Based on these evidence, it was concluded that ARNT/HIF-1β is indeed one of the key players regulating the transcriptional network of pancreatic beta-cells and that it may play an important role in the pathogenesis of T2D.

Although ARNT/HIF-1β was identified as important for maintaining beta-cell functional competence and normal glucose homeostasis in mice, we did not know the identity of the key metabolic pathways that are regulated by the transcription factor which are critical for glucose-stimulated insulin secretion. Our goal was to investigate the metabolic consequence of loss of ARNT/HIF-1β in pancreatic beta-cells using the INS-1 derived 832/13 beta-cell line where ARNT/HIF-1β expression was knocked down by an siRNA. In agreement with Gunton et al, we found that reduction of ARNT/HIF-1β expression decreases GSIS in the 832/13-cell line. We also found that loss of ARNT/HIF-1β affects glucose utilization without affecting glucose oxidation or ATP/ADP ratio. Our results indicate the novel finding that ARNT/HIF-1β regulates glucose-stimulated anaplerosis and it is necessary for the glucose-induced rise in fatty acids and that the mechanism of ARNT/HIF-1β-mediated insulin release is independent of beta-cell ATP production.

3.3 Contributions

Gas chromatography-mass spectrometry (GC-MS) based analysis of the metabolites was done in collaboration with Mei Huang, PhD candidate in Dr. Jamie Joseph's lab. 832/13 cells were seeded, transfected and prepared for GC-MS by Renjitha Pillai. Samples were run on GC-MS and data analyzed by Mei Huang.

3.4 Results

3.4.1 siRNA Mediated Knock-Down of ARNT/HIF-1β in 832/13 Cell Line

832/13 cells were transfected with one of the two siRNA duplexes targeting two different regions of the ARNT/HIF-1β gene transcript namely, siARNT1 and siARNT2 (see section 2.2 under "Experimental Procedures" for sequence details) or a control, nonspecific siRNA sequence (siControl). 72 h post transfection, cells were harvested to measure ARNT/HIF-1β RNA and protein expression levels by real time PCR and Western blot, respectively. Treatment of 832/13 cells with siARNT1 resulted in a 78 ± 4% reduction of ARNT/HIF-1β mRNA as compared with the 56 ± 5% knockdown achieved by siARNT2. The effect on ARNT/HIF-1β mRNA knockdown mediated through siARNT1 was found to be statistically

more significant than mediated through siANRT2 ($p<0.01$, siARNT1 vs siARNT2, student's t-test) (Figure 7A). ARNT/HIF-1β protein levels in 832/13 cells treated with siARNT1 were reduced by 67 ± 15% (Figure 7 B, C).

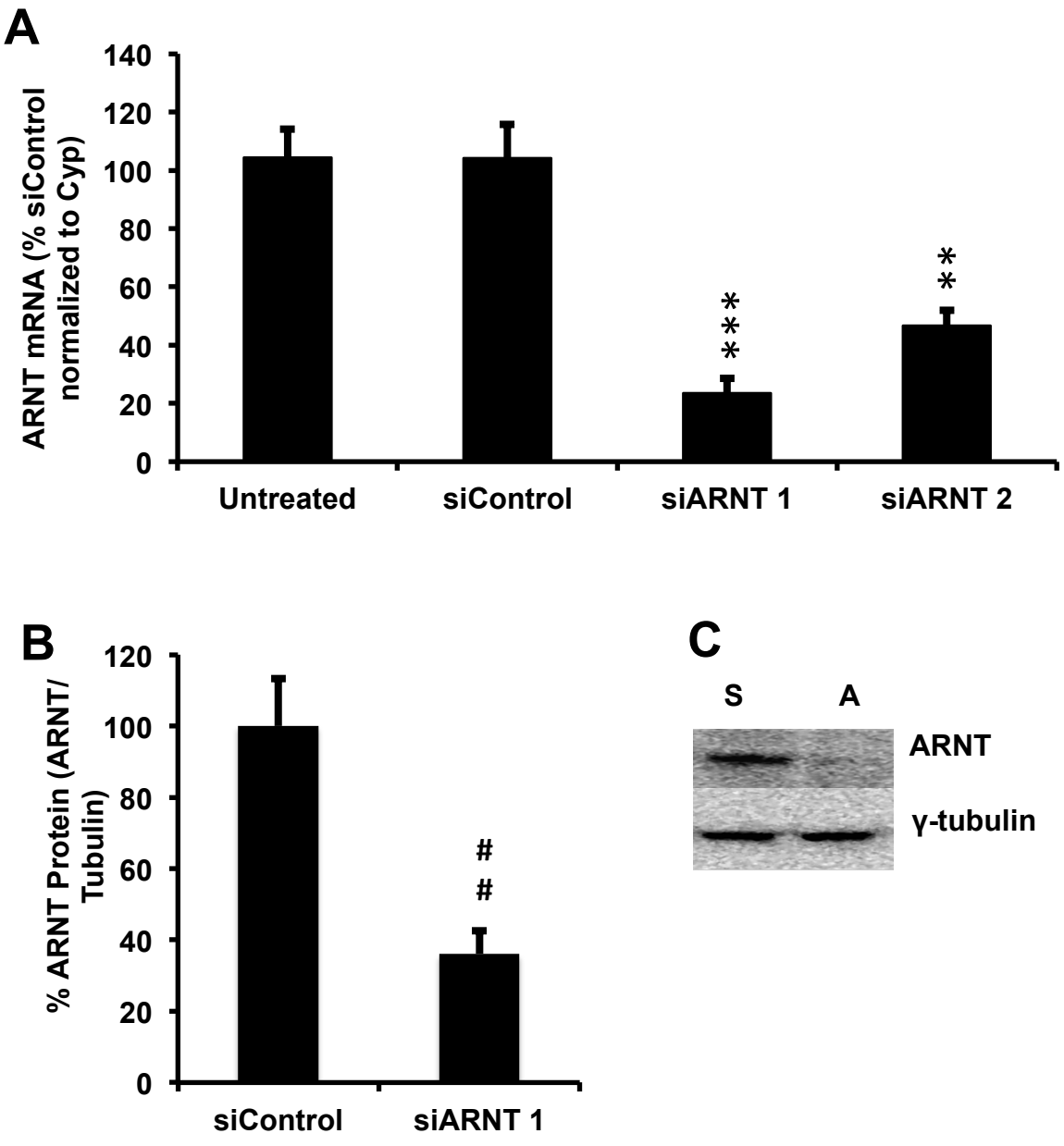

Figure 7: Effects of siARNT1 and siARNT 2 duplexes on gene expression and protein levels in 832/13 cells. A) Both siARNT 1 and siARNT2 decrease ARNT/HIF-1β mRNA levels. B, C) Immuno blot analysis of extracts from 832/13 cells-treated with siControl (S) and siARNT 1 (A). Results represent mean ± SEM from 3 - 6 experiments. ***p<0.01, ***p<0.001 siControl Vs siARNT, ANOVA post hoc Tukey's test. ## p <0.01 siControl Vs siARNT, Student's t-test.*

3.4.2 ARNT/HIF-1β Suppression Impairs GSIS in 832/13 Cells

832/13 cells were either left untreated or transfected with siControl, siARNT1, or siARNT2 duplexes. After 72 h, GSIS was performed by pre-incubating cells for 2 h at low glucose concentrations (2 mM) followed by stimulating the cells with either low glucose (2 mM) or high glucose (16.7 mM) for the next 2 h. Insulin release at high glucose was significantly inhibited by 60 ± 10% for siARNT1 and by 52 ±17% for siARNT2 as compared with the siControl (Figure 8). These results were consistent with the results obtained by Gunton et al where GSIS was inhibited in MIN6 cells treated with siRNA against ARNT/HIF-1β (164). Insulin secretion was normalized to total protein content.

NOTE: Although the impact of siARNT1 and siARNT2 on GSIS was not statistically different, based on the gene expression analysis, siARNT1 inhibited ARNT/HIF-1β gene expression significantly more compared to siARNT2. Hence siARNT1 was used for all subsequent gene knock down experiments and from here on it will be denoted as siARNT.

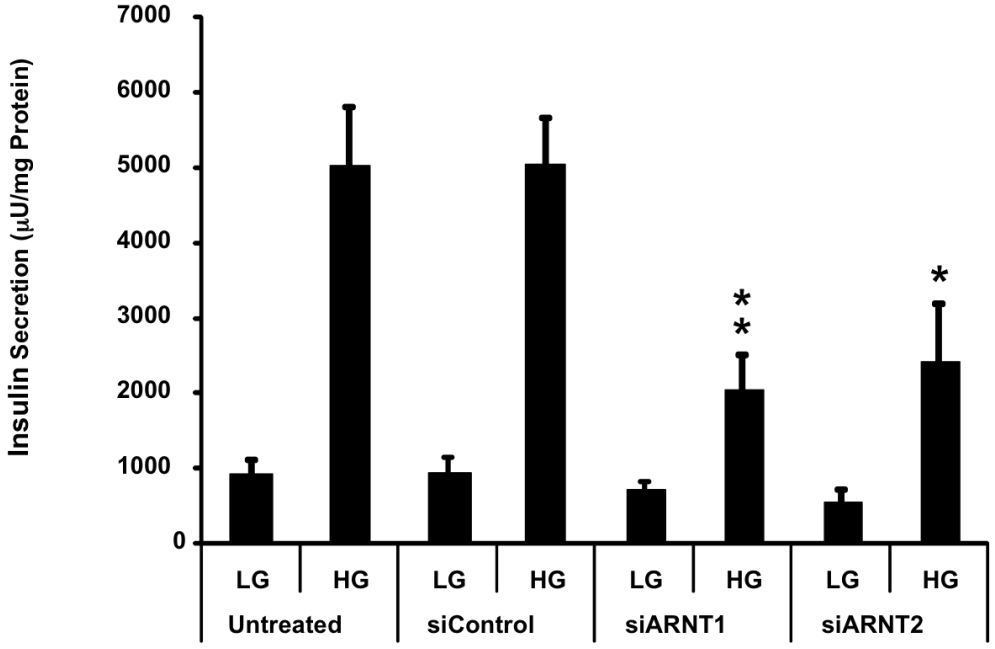

Figure 8: Effect of siARNT 1 and siARNT 2 on glucose-stimulated insulin secretion in 832/13 cells. LG, low glucose (2 mM); HG, high glucose (16.7 mM); Compared to siControl, both siARNT 1 and siARNT inhibited GSIS in 832/13 cells. Results represent mean ± SEM from 3 - 6 experiments. **p<0.05, **p<0.01 HG siControl Vs HG siARNT. Anova, post hoc Tukey's test.*

3.4.3 ARNT/HIF-1β Suppression Impairs Glucose Utilization without Affecting Glucose Oxidation in 832/13 Cells

Glucose utilization and oxidation were assessed to evaluate changes in glucose metabolism following ARNT/HIF-1β knockdown. ARNT/HIF-1β is a heterodimeric transcription factor necessary to mediate cellular responses under low oxygen conditions (96). In this capacity, ARNT/HIF-1β was shown to activate the expression of key glycolytic enzymes and based on this we hypothesized that loss of ARNT/HIF-1β may lead to impairment in glucose utilization, which may result in concomitant changes in glucose oxidation.

As expected, when glucose was raised from 2 mM to 16.7 mM, siControl cells exhibited significant increase in glycolytic flux as measured by [5-^{3}H] glucose usage assay. Differences in glucose utilization were not statistically different at 2 mM glucose between siControl and siARNT cells (Figure 9A). However, in the presence of 16.7 mM glucose the glycolytic flux was significantly reduced by 31 ± 6% in siARNT-treated cells compared to siControl-treated cells.

As for glucose oxidation, when glucose was raised from 2 mM to 16.7 mM, siControl cells exhibited a significant increase in glucose oxidation as measured by the CO_2 production by [U-^{14}C] glucose. Although glycolytic flux was negatively affected by ARNT/HIF-1β knockdown at 16.7 mM glucose, we could not detect significant changes in glucose oxidation at both 2 mM and 16.7 mM glucose in siARNT-treated cells (Figure 9B).

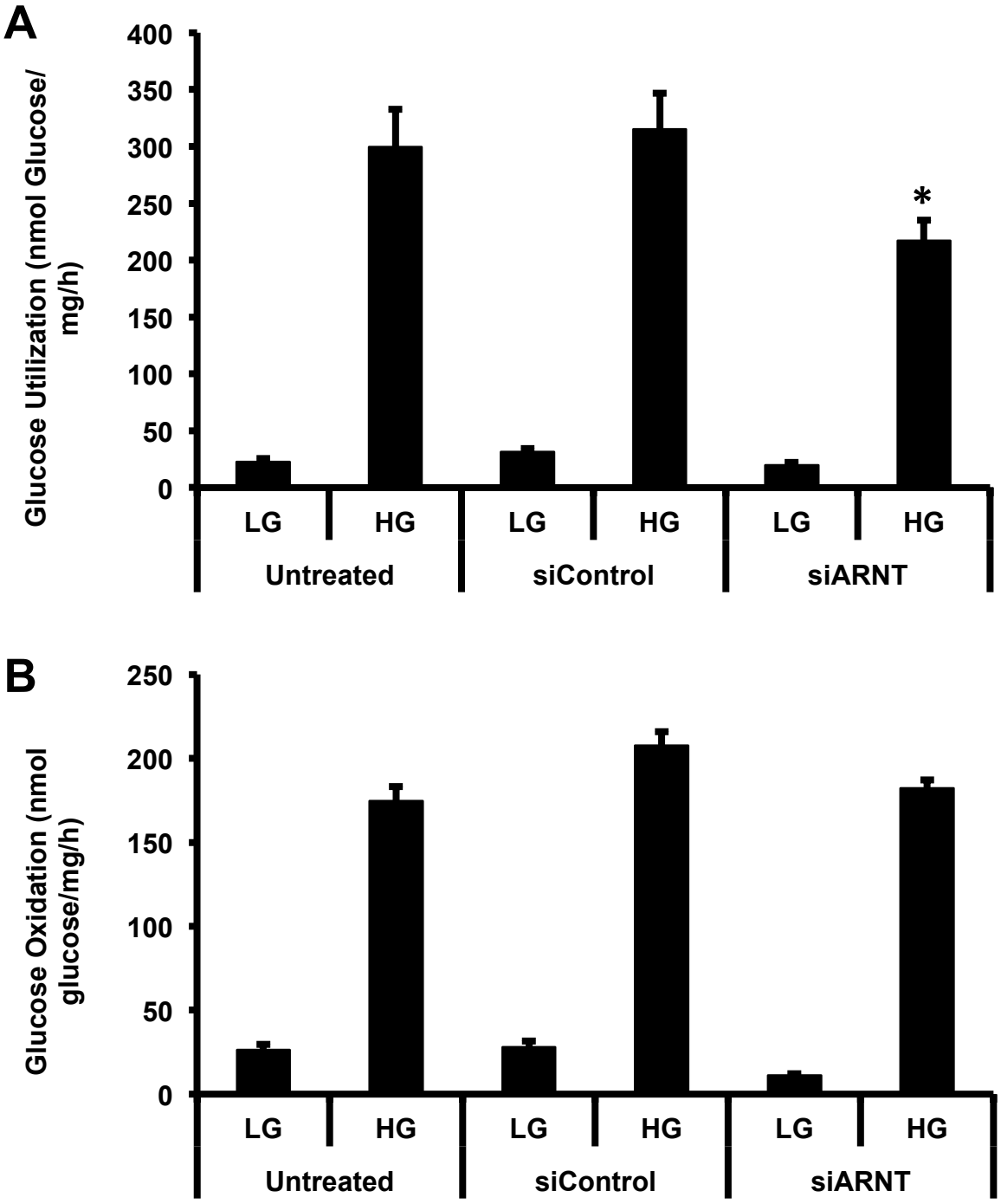

Figure 9: Effects of siARNT on glucose utilization and glucose oxidation in 832/13 cells. A) glucose utilization. B) glucose oxidation. LG, low glucose (2 mM); HG, high glucose (16.7 mM). Data represent mean ± SEM. of 6 - 8 independent experiments. **p < 0.05 HG untreated Vs HG siControl Vs HG siARNT, Anova, post hoc Tukey's test.*

3.4.4 ARNT/HIF-1β Suppression Does Not Significantly Alter the Glucose-Induced Changes in the ATP/ADP Ratio in 832/13 Cells

Glucose-stimulated ATP production is one of the most critical signals coupling metabolism of glucose to insulin granule exocytosis. Although there was a reduction in glycolytic flux by 31% in siARNT-treated cells, glucose oxidation was not affected suggesting that glucose-stimulated ATP levels may not be altered in siARNT-treated cells. We observed a slight reduction in both ATP levels and the ATP/ADP ratio following ARNT/HIF-1β knockdown, however, this was not statistically different from that observed in siControl-treated 832/13 cells (Figure 10A, B). Our ATP data is consistent with the observation that glucose oxidation remained unaltered in the absence of ARNT/HIF-1β in 832/13 cells.

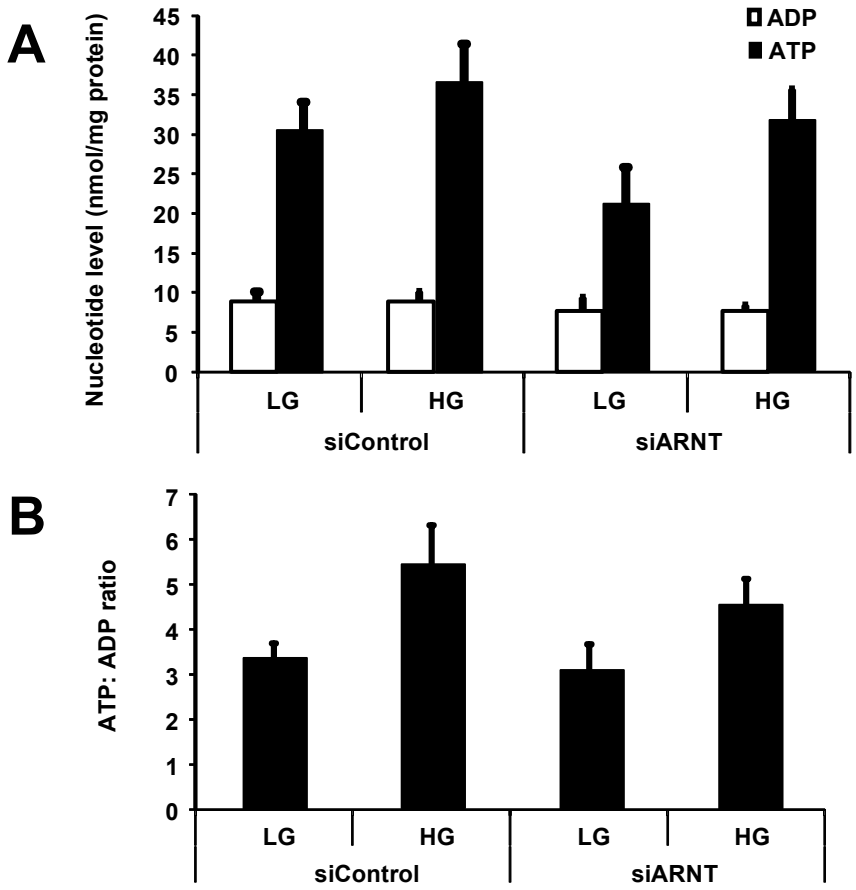

Figure 10: Effects of siARNT on A) ADP and ATP amount B) ATP:ADP ratio in 832/13 cells. *LG,* low glucose (2 mM); *HG,* high glucose (16.7 mM). Data represents mean ± SEM. of 6 independent experiments.

3.4.5 GC-MS-based Metabolic Profiling of Glycolysis and TCA Intermediates in 832/13 Cells with Reduced ARNT/HIF-1β Levels

To further understand the key metabolic pathways affected by ARNT/HIF-1β knockdown, a GC-MS based metabolomics approach was employed. Assessment of the metabolic profile of the glycolytic, pentose phosphate, TCA cycle, free fatty acid and amino acid pathways in both siControl and siARNT-treated cells was performed using an Agilent GC-MS system. This comparative analysis revealed that levels of most of the glycolytic (Figure 11) and TCA cycle intermediates (Figure 12) were markedly reduced by ARNT/HIF-1β knockdown. Glycolytic intermediates such as dihydroxyacetone phosphate, 3-phosphoglycerate and pyruvate were significantly reduced in siARNT-treated cells exposed to 16.7 mM glucose. However, the amount of lactate was significantly down at both 2 mM and 16.7 mM in siARNT-treated cells. Glucose-stimulated rise in glucose 6-phosphate levels was not affected by ARNT/HIF-1β knockdown, suggesting that glucokinase activity is preserved in siARNT-treated cells. These findings are in agreement with the observation that glycolytic flux is markedly reduced by ARNT/HIF-1β knockdown at 16.7 mM glucose.

Glucose-stimulated rise in the levels of TCA intermediates were significantly lower in siARNT-treated cells compared to siControl-treated cells (Figure 12). Levels of citrate/isocitrate, α-ketoglutarate, succinate, fumarate and malate were significantly lower in siARNT-treated cells in the presence of 16.7 mM glucose. Among the TCA intermediates, the reduction in metabolite levels following ARNT/HIF-1β knockdown was most notable for α-ketoglutarate (90 ± 5%), followed by succinate (68 ± 2%), fumarate (67 ± 1%), malate (65 ± 2%) and citrate/isocitrate (49 ± 8%). In addition, this marked difference in the amount of TCA metabolites was also significantly reduced at low glucose concentrations (2 mM). The fact that glucose oxidation was not significantly affected under high glucose conditions, suggests that the amounts of TCA metabolites seen in siARNT-treated cells are likely sufficient for maintaining glucose oxidation and preservation of cell viability. The reduced amounts of malate, citrate, and isocitrate are of direct interest in the regulation of GSIS because these metabolites act as intermediates in the pyruvate cycling pathways and

generation of NADPH, which have been suggested to play a key role in GSIS (67,70,76,79,91,218). They also serve as a source of glucose carbon for lipogenesis, which has also shown to be important for GSIS (75,219-221).

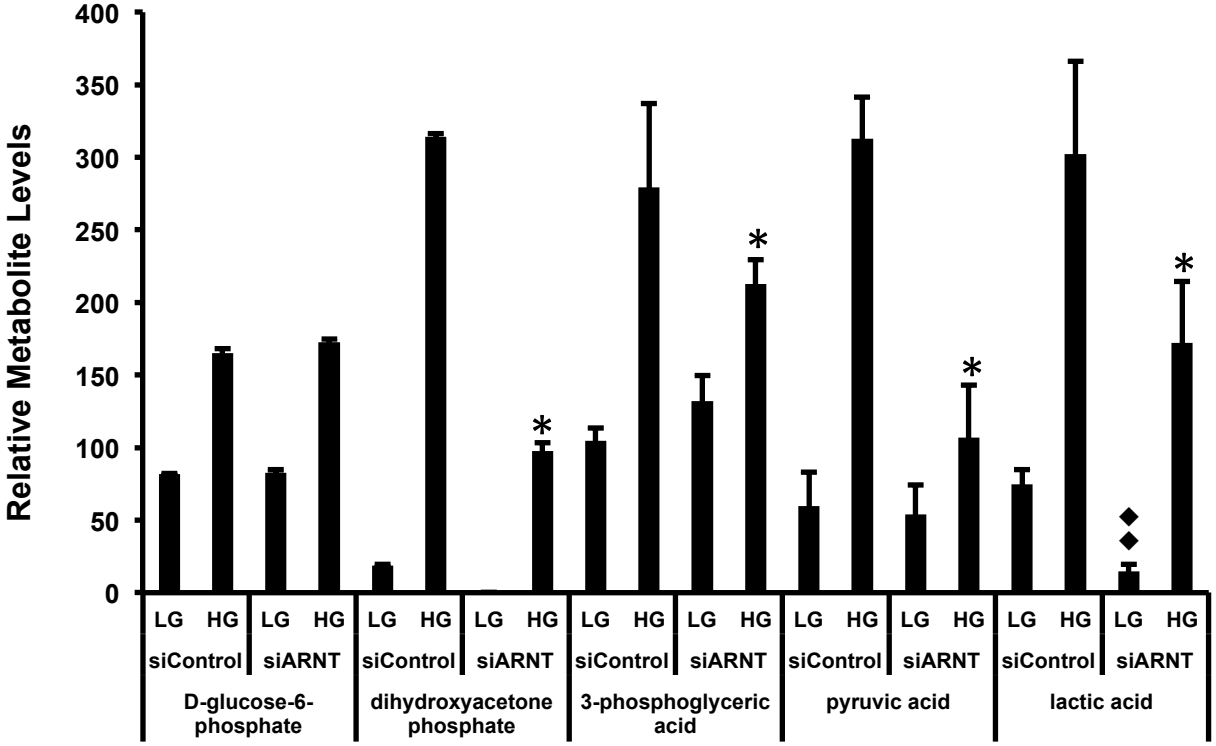

Figure 11: Effects of siARNT on glycolytic metabolite levels in 832/13 cells. Data presented as a ratio of target metabolite corrected for an internal control (myristic acid-d27) and represents mean ± SEM of 3 independent experiments. *LG,* low glucose (2 mM); *HG,* high glucose (16.7 mM). **, $p < 0.05$, HG siControl Vs HG siARNT; ◆◆$p < 0.01$, LG siControl Vs LG siARNT. Student's t-test.*

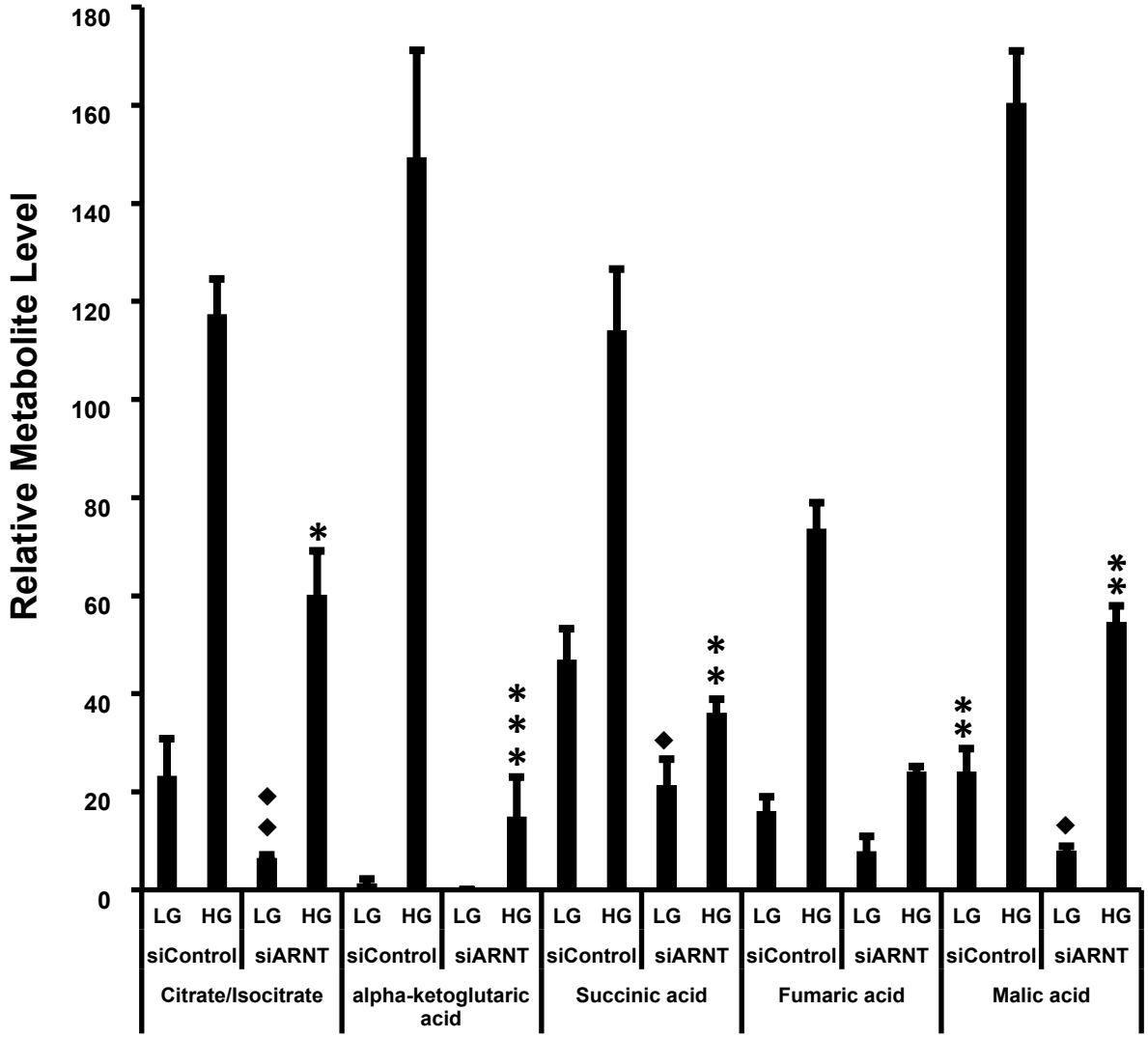

Figure 12: Effects of siARNT on TCA metabolite levels in 832/13 cells. Data presented as a ratio of target metabolite corrected for an internal control (myristic acid-d27) and represents mean± SEM of 3 independent experiments. *LG,* low glucose (2 mM); *HG,* high glucose (16.7 mM). *p<0.05, ***p < 0.01, ****p < 0.001 HG siControl Vs HG siARNT; ◆p < 0.05, ◆◆p < 0.05, LG siControl Vs LG siARNT. Student's t-test.*

3.4.6 GC-MS-based Metabolic Profiling of Pentose Phosphate Pathway (PPP) Intermediates in 832/13 Cells with Reduced ARNT/HIF-1β Levels

Although the role of PPP in GSIS is still highly debated, some studies have shown that PPP plays a critical role in the generation of NADPH and that activation of PPP is necessary for scavenging reactive oxygen species (ROS) produced as a result of chronic exposure to high glucose (222,223). Upon stimulation with 16.7 mM glucose, levels of 6-phosphogluconate were similar in siARNT-treated cells compared to siControl-treated cells indicating that the amount of glycolytic substrate entering the PPP under these conditions is preserved although glycolysis itself was impaired in the absence of ARNT/HIF-1β (Figure 13). ARNT/HIF-1β knockdown led to a reduction in ribose 5-phosphate levels when exposed to 16.7 mM glucose. The final step in the oxidative phase of PPP does not seem to be affected by ARNT/HIF-1β silencing as glucose-induced rise in ribulose 5-phosphate levels were similar in siARNT-treated cells compared to siControl-treated cells. In conclusion, we observed that the non-oxidative branch of PPP was more affected by ARNT/HIF-1β knockdown than the oxidative branch.

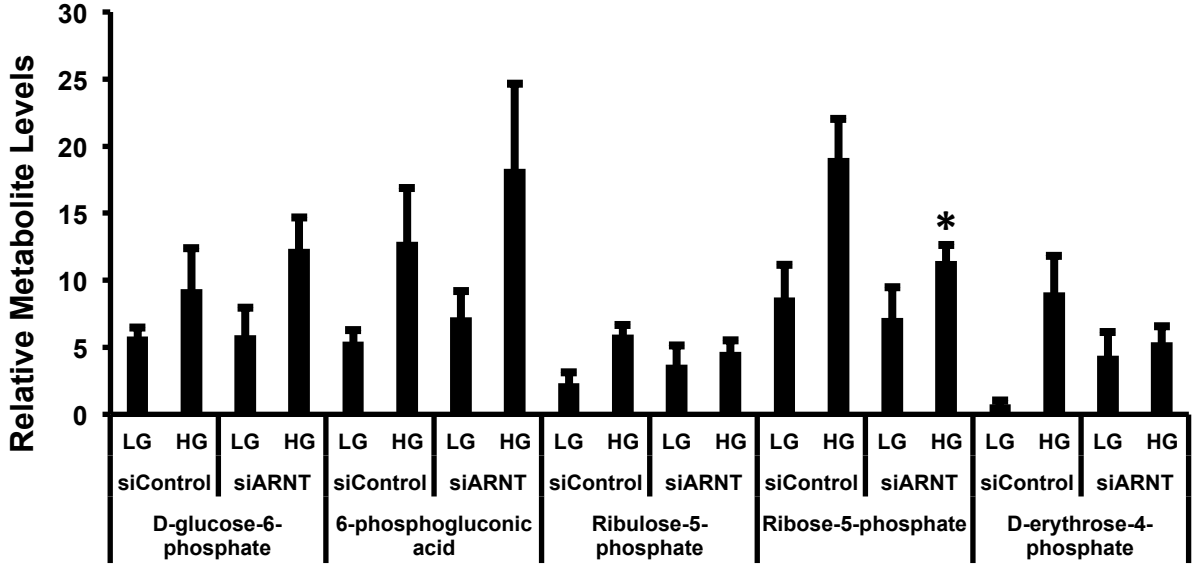

Figure 13: Effects of siARNT on pentose phosphate pathway in 832/13 cells. Data presented as a ratio of target metabolite corrected for an internal control (myristic acid-d27) and represents mean ± SEM of 3 independent experiments. *LG,* low glucose (2 mM); *HG,* high glucose (16.7 mM). **p < 0.05 HG siControl Vs HG siARNT. Student's t-test.*

3.4.7 GC-MS-based Metabolic Profiling of Free Fatty Acids and Amino Acids in 832/13 Cells with Reduced ARNT/HIF-1β Levels

Glucose-induced rise in fatty acid production associated with the rise in glucose concentration from basal (2 mM) to stimulatory concentration (16.7 mM), was completely absent in siARNT-treated cells compared to siControl-treated cells. The levels of fatty acid species including palmitic acid, palmitoleic acid, oleic acid and stearic acid were significantly reduced in siARNT-treated cells under high glucose concentrations (16.7mM), indicating that mitochondrial export of citrate to the cytosol, critical for lipogenesis could be diminished in siARNT-treated cells under high glucose conditions.

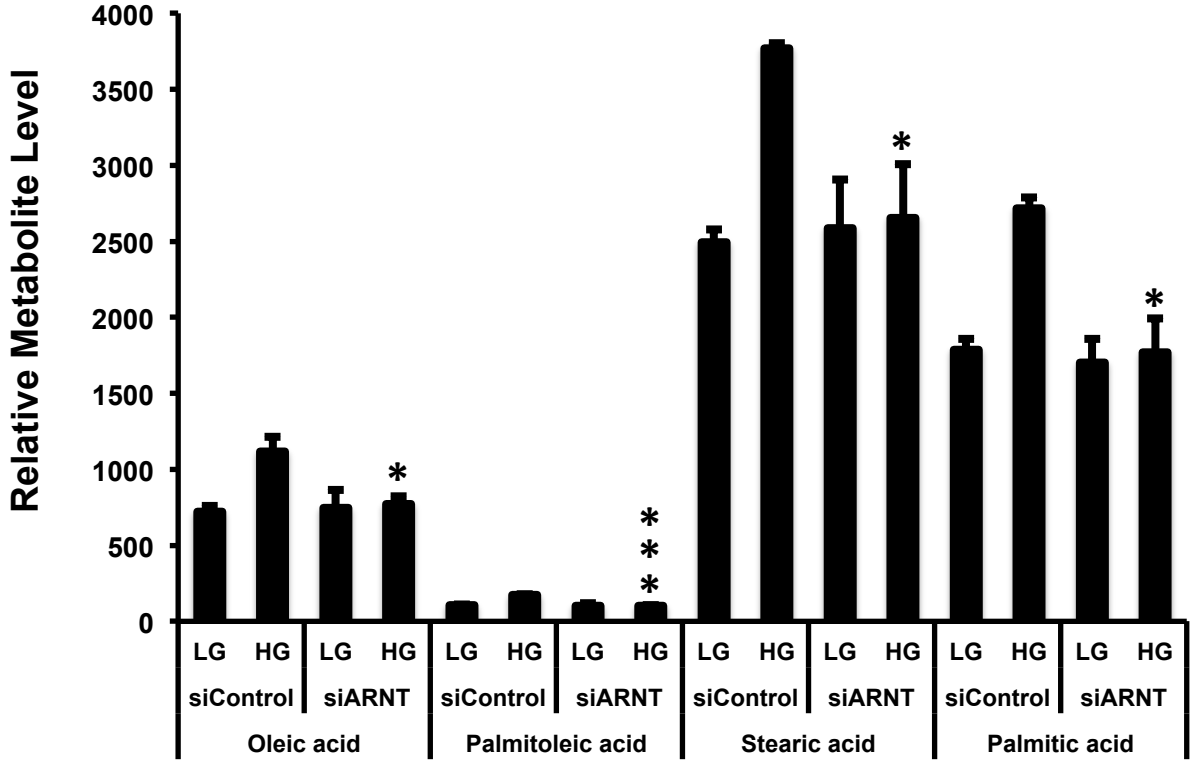

Figure 14: Effects of siARNT on glucose induced fatty acid production in 832/13 cells. Data presented as a ratio of target metabolite corrected for an internal control (myristic acid-d27) and represents mean ± SEM of 3 independent experiments. *LG,* low glucose (2 mM); *HG,* high glucose (16.7 mM). **p < 0.05, ***p < 0.001 HG siControl Vs HG siARNT. Student's t-test.*

Finally, the impact of ARNT/HIF-1β knockdown also seems to extend into amino acid metabolism but to a lesser extent. Only a few amino acids were affected in siARNT-treated cells as the levels of L-glutamate, L-threonine and L-alanine were significantly lowered at high glucose concentrations (16.7 mM) (Figure 15). The discovery that L-glutamate production is diminished in siARNT-treated cells is interesting as it represents another potential candidate coupling factor that is believed to play important signalling roles in GSIS (68,203).

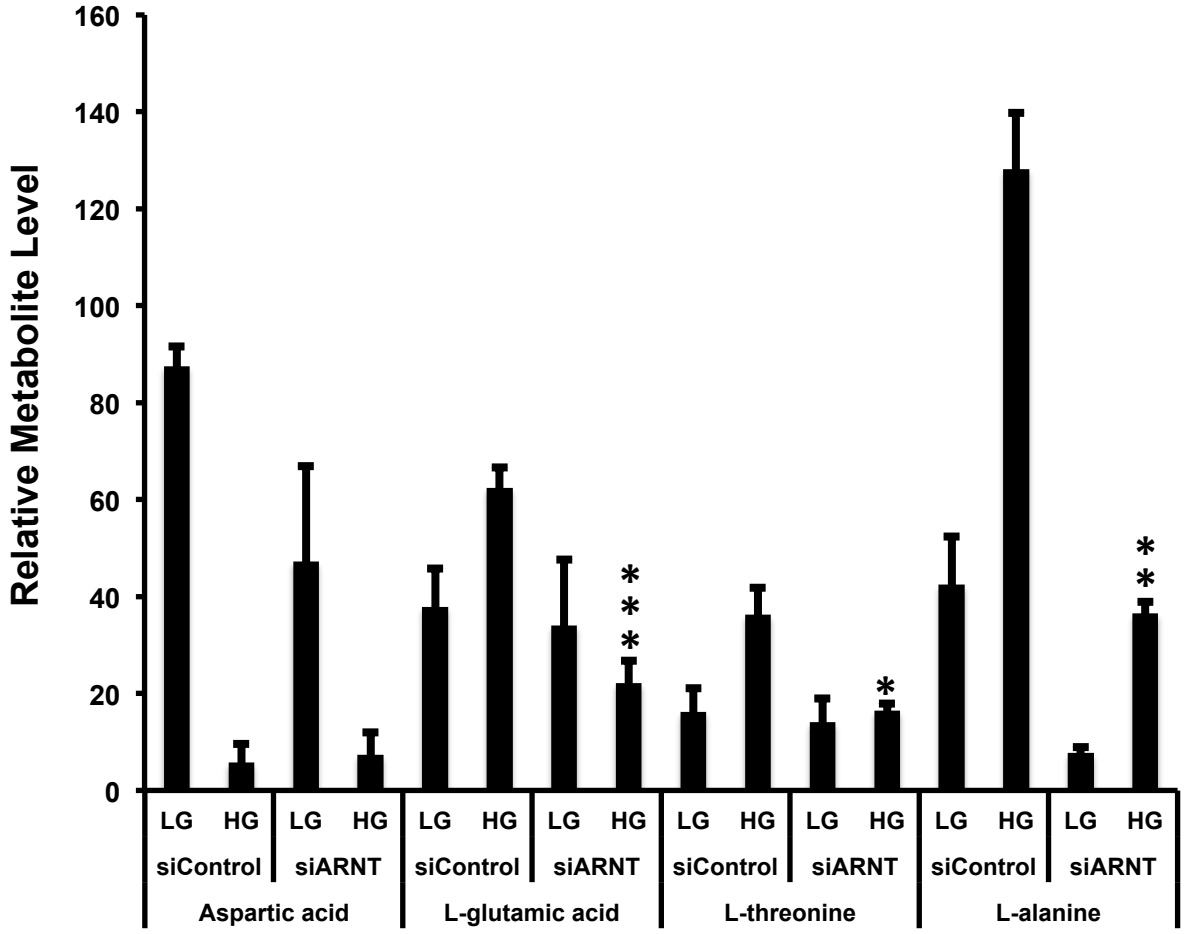

Figure 15: Effects of siARNT on amino acid levels in 832/13 cells. Data presented as a ratio of target metabolite corrected for an internal control (myristic acid-d27) and represents mean ± SEM of 3 independent experiments. *LG,* low glucose (2 mM); *HG,* high glucose (16.7 mM). **p < 0.05, **p < 0.01, ***p < 0.001 HG siControl Vs HG siARNT. Student's t-test.*

3.4.8 Gene Expression Profiling of 832/13 Cells with Reduced ARNT/HIF-1β Levels

In an attempt to understand the molecular mechanism involved in the regulation of GSIS by ARNT/HIF-1β, we assessed the expression of those transcription factors that are critical for maintaining beta-cell function in 832/13 cells treated with siARNT (Figure 16 A). Hypoxia-inducible factor-1α (HIF-1α), the heterodimeric binding partner for ARNT/HIF-1β, was used as a positive control in 832/13 cells as previous studies have shown that the expression of HIF-1α was independent of ARNT/HIF-1β (163). Expression of genes that are known to be important for maintaining normal beta-cell function such as the hepatocyte nuclear factor-1α (HNF-1α), HNF-4α, peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) and guanine exchange protein activated by cAMP, Epac 2, was significantly reduced in response to siRNA-mediated reduction of ARNT/HIF-1β. This data suggests that ARNT/HIF-1β acts upstream of these transcription factors in beta-cells.

Next, we assessed the expression of genes that are critical for maintaining normal glucose metabolism in siARNT-treated 832/13 cells (Figure 16 B). Particularly, the expression of genes that are involved in glucose entry and phosphorylation namely, glucose transporter 2 (GLUT2) and glucokinase (GK), were significantly reduced in 832/13 cells with reduced ARNT/HIF-1β levels. Expression of genes that play a critical role in the maintenance of mitochondrial glucose metabolism, specifically the oxidative and the anaplerotic enzymes, namely, pyruvate dehydrogenase α1 (PDHα1) and pyruvate carboxylase (PC), were both found to be significantly reduced in siARNT-treated 832/13 cells. PDH expression was found to be less impacted than PC in siARNT-treated cells. PDHα1 is important for the generation of NADH and ATP and PC is important for the anaplerotic entry of pyruvate into the mitochondria. Export of mitochondrial metabolites to the cytosol occurs via metabolite carriers namely dicarboxylate carrier (DIC; malate transporter), 2 oxoglutarate carrier (OGC; α-ketoglutarate transporter), and citrate carrier (CIC; citrate transporter). The optimal expression of these carriers is critical for maintaining glucose-stimulated anaplerosis and GSIS in beta-cells (88,224,225). Interestingly, both DIC and OGC were significantly reduced

in the absence of ARNT/HIF-1β. Reductions in PC, DIC, and OGC in the absence of ARNT/HIF-1β are in agreement with our metabolomics data showing a reduction in anaplerosis in siARNT-treated cells. Expression of two key enzymes involved in the proposed pyruvate cycling pathways, namely, cytosolic $NADP^+$-dependent malic enzyme (MEc) and cytosolic $NADP^+$-dependent isocitrate dehydrogenase (ICDc) was also studied. (87,91,226). Only MEc and not ICDc expression was significantly down regulated in siARNT-treated cells.

Based on our metabolomics data, the levels of certain free fatty acids were lower in siARNT-treated cells and therefore, we also assessed the expression of key fatty acid metabolism enzymes (Figure 17). Expression of acetyl CoA synthase (ACS), an enzyme that is required for the formation of the precursor molecule, acetyl CoA, for fatty acid synthesis was found to be unaffected by siARNT treatment. We also looked at the expression of enzymes such as citrate lyase (CL), fatty-acid synthase (FAS) and carnitine palmitoyltransferase 1α (CPT1α) in the absence of ARNT/HIF-1β. Both FAS and CPT1α were significantly lower in siARNT-treated cells compared to siControl cells. Expression of CL, which is critical for *de novo* lipogenesis, was found to be significantly reduced in the absence of ARNT/HIF-1β in 832/13 cells.

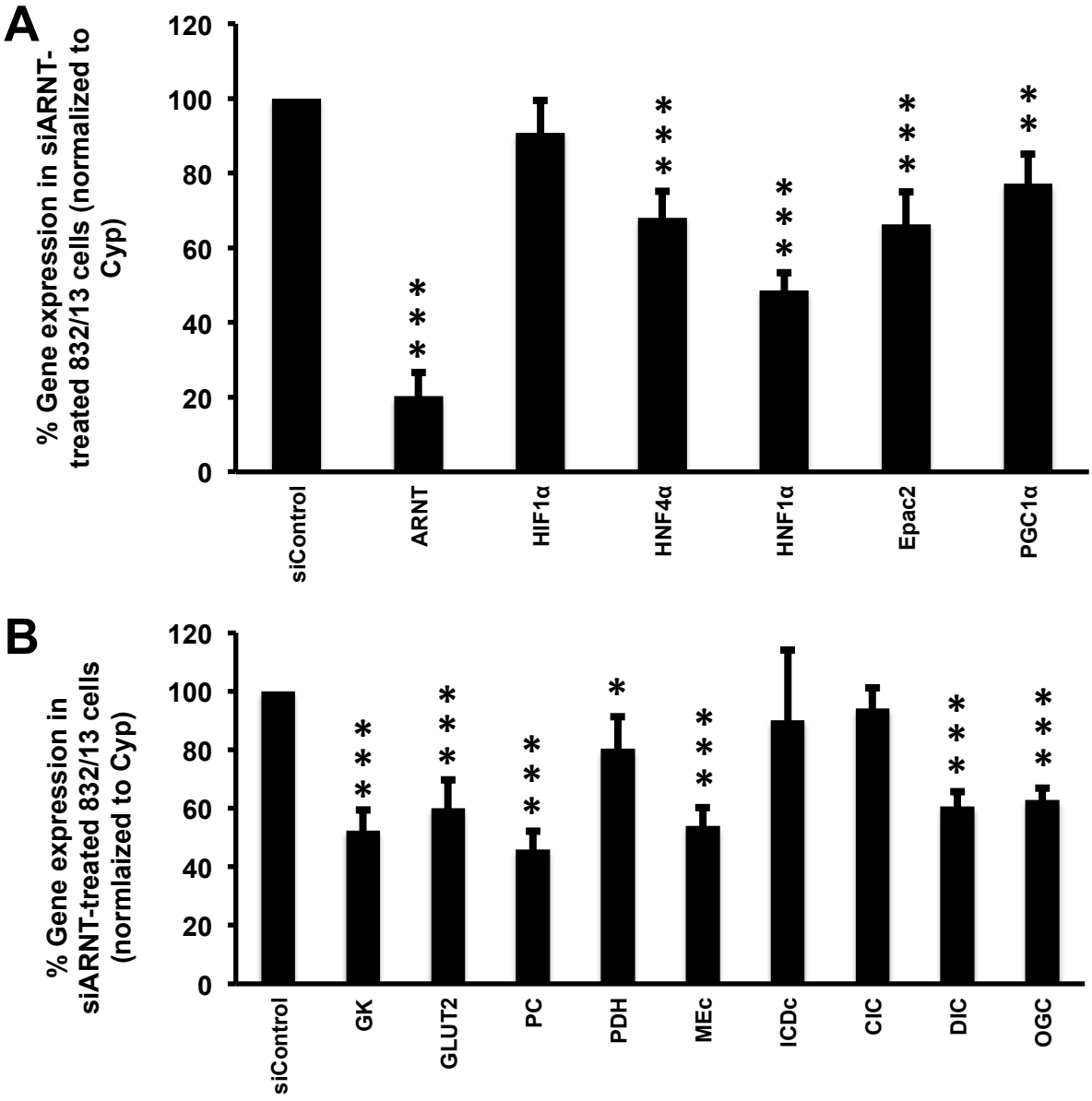

Figure 16: **Effects of siRNA-mediated suppression of ARNT/HIF-1β on key genes involved in maintaining beta-cell function 832/13 cells.** Gene expression is expressed as a percentage of the target gene from siControl-treated cells and corrected by an internal control gene cyclophilin E (Cyp). There was no significant difference seen between treatment groups for cyclophilin E. A) HIF1α, hypoxia-inducible factor 1α; HNF4a, hepatocyte nuclear factor-4α; HNF1a, hepatocyte nuclear factor-1α; Epac 2, Guanine exchange protein, PGC 1α, peroxisome proliferator-activated receptor-gamma coactivator 1α; B) GK, glucokinase; GLUT2, glucose transporter-2; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; MEc, cytosolic malic enzyme; ICDc, cytosolic isocitrate dehydrogenase; CIC, citrate carrier; DIC, dicarboxylate carrier; OGC, α-ketoglutarate carrier; Data represents mean ± SEM. of 7 independent experiments. **p<0.05, **p<0.01, ***p<0.001 siControl Vs siARNT. Student's t-test.*

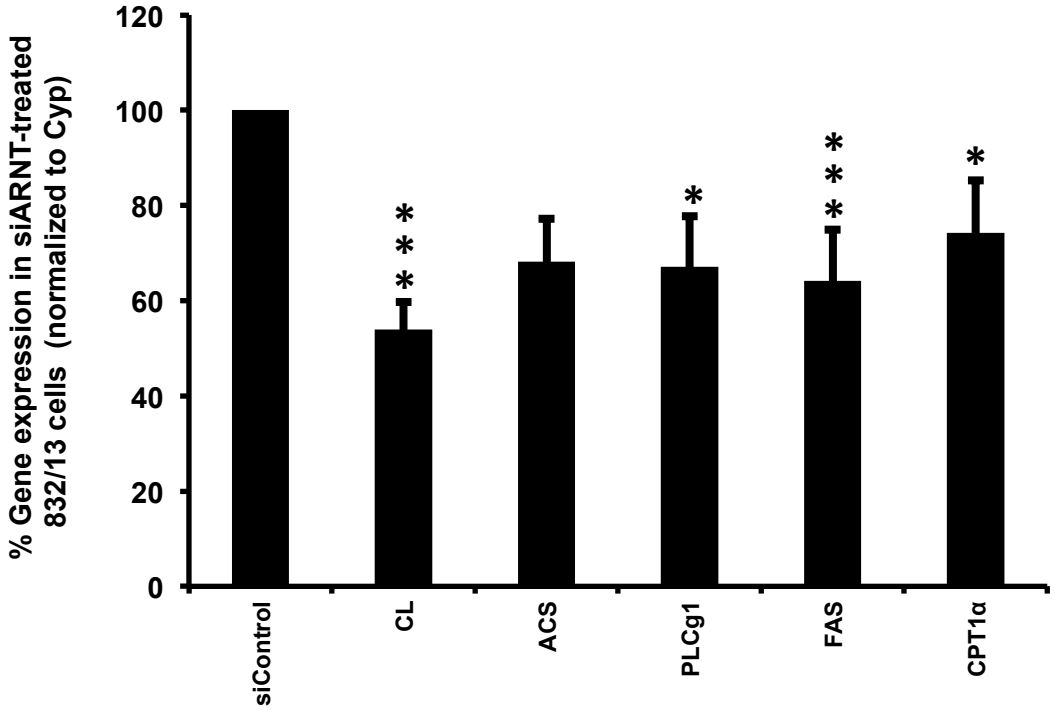

Figure 17: **Effects of siRNA-mediated suppression of ARNT/HIF-1β on key genes involved in fatty-acid synthesis in 832/13 cells.** Gene expression is expressed as a percentage of the target gene from siControl-treated cells and corrected by an internal control gene cyclophilin E (Cyp). Cl, citrate lyase; ACS, acetyl CoA synthase, PLCg1, phopholipase C-gamma 1, FAS, fatty-acid synthase; CPT1α, carnitine palmitoyltransferase 1α. Data represents mean ± SEM of 7 independent experiments. **p<0.05, ***p<0.001 siControl Vs siARNT. Student's t-test.*

3.4.9 Effects of Amino Acids, Fatty Acids and IBMX on Insulin Secretion in the Absence of ARNT/HIF-1β in 832/13 cells

A variety of signals generated either through the metabolism of glucose or other non-carbohydrate nutrients like amino acids and fatty acids are involved in the acute stimulation of insulin secretion from the pancreatic beta-cells. In an effort to determine whether these additional pathways are functional in beta-cells deficient in ARNT/HIF-1β, siARNT-treated 832/13 cells were subjected to further investigation using various insulin secretagogues.

Both leucine and glutamine are known to increase insulin secretion in 832/13 cells as well as in islets in the absence of glucose. Stimulation of insulin secretion by leucine is through the allosteric activation of glutamate dehydrogenase whereas glutamine provides carbons to the TCA cycle (227-229). In order to assess the impact of ARNT/HIF-1β knockdown on amino acid stimulated insulin secretion, we tested the effects of a mixture of both leucine and glutamine (both at 2 mM and 10 mM each) on insulin secretion on 832/13 cells deficient in ARNT/HIF1β. As shown in Figure 18 A, the combination of two amino acids increased insulin secretion in the absence of glucose under all conditions and no significant difference was observed between the siControl and siARNT-treated cells at both 2 mM and 10 mM amino acid concentrations.

The role of GLP-1 in beta-cell survival and function mediated through the cAMP signalling pathway is a well-studied phenomenon in beta-cells (230,231). Increase in intracellular cAMP leads to the activation of protein kinase A (PKA), which then phosphorylates and activates a number of beta-cell proteins like cAMP response element binding protein (CREB), which in turn regulates the expression of key genes that are important for beta-cell survival and insulin secretion. In addition, increase in intracellular cAMP concentration has been found to increase the accumulation of HIF-1α, the heterodimeric binding partner for ARNT/HIF-1β in pancreatic beta-cells (232). In an attempt to understand whether ARNT/HIF-1β mediates its effect on GSIS through the incretin pathway, we decided to study insulin secretion in response to 3-isobutyl-1-methylxanthine (IBMX), a non-specific phosphodiesterase (PDE) inhibitor, which raises the intracellular cAMP level and activates PKA (233). Insulin secretion was measured in response to 50 μM IBMX in the presence of both 2 mM and 7 mM glucose (Figure 18 B). In the presence of 2 mM glucose, we observed a mild increase in insulin secretion in both siControl and siARNT-treated cells, however, this was not significantly different between the two groups. In the presence of 7 mM glucose, IBMX clearly increased insulin secretion by 2 fold in siControl cells and the potentiating effect of IBMX was clearly visible in siARNT-treated cells as well. However, glucose induced increments in insulin secretion were significantly lower and reduced by about 56%

in siARNT-treated cells compared to siControl-treated cells, suggesting that defects in insulin secretion observed in the absence of ARNT/HIF-1β are specific to glucose.

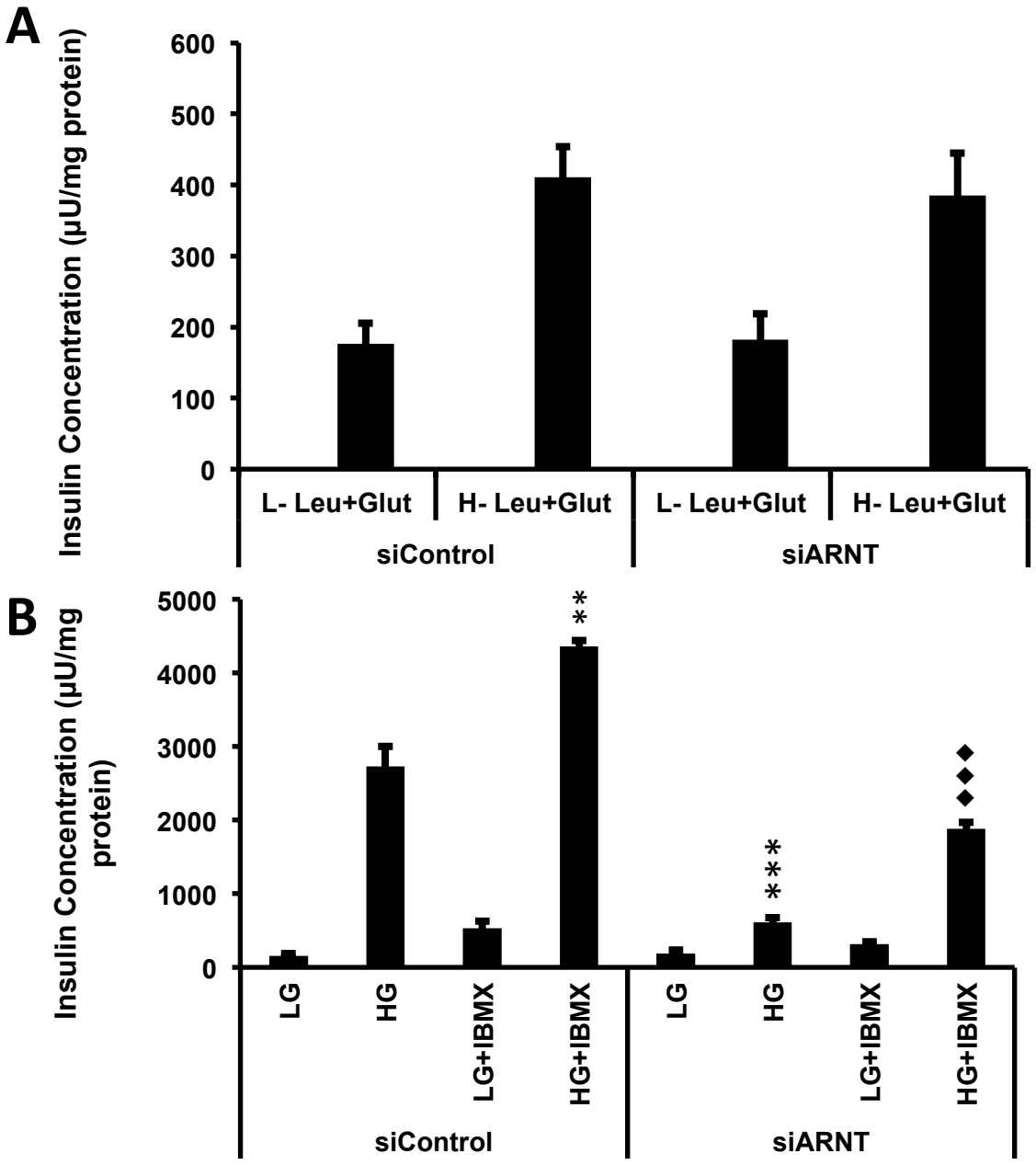

Figure 18: Effects of amino acids and IBMX on insulin secretion in siARNT-treated 832/13 cells. A) Amino acids, Leucine (Leu) + Glutamine (Glut), L-low, 1 mM each of Leu+ Glut; H- high, 10 mM each of Leu +Glut B) IBMX, 3-isobutyl-1-methylxanthine (50 µM) LG, low glucose (2 mM); HG, high glucose (7 mM). Data represents mean ± SEM of 4 independent experiments. ***p<0.01 for HG siControl Vs HG+IBMX siControl, ***p<0.001 siControl HG Vs siARNT HG, ◆◆◆p < 0.001 siControl HG+IBMX Vs siARNT HG+IBMX. Student's t-test.*

The ability of fatty acids to potentiate insulin secretion acutely in the presence of glucose is well studied (221,234,235). Several mechanisms were proposed to explain the effectiveness of fatty acids on insulin secretion including the binding of free fatty acids to cell surface receptor GPR40, leading to phospholipase C activation, IP_3 production and rise in intracellular Ca^{2+} (236-238). To see whether acute stimulation by fatty acids is effective in 832/13 cells treated with siARNT, we exposed the cells to 250 μM palmitate, a well studied saturated fatty acid (C16:0), abundantly found in the plasma. Insulin secretion in response to palmitate was measured in the presence of both 2 mM and 7 mM glucose in 832/13 cells treated with siARNT (Figure 19 A). As expected, the potentiating effects of palmitate were absent in the presence of 2 mM glucose. We observed that palmitate acutely stimulated insulin secretion in the presence of 7 mM glucose in both siControl and siARNT-treated cells, however, the extent of stimulation was around 25% less in siARNT-treated cells compared to siControl cells in the presence of glucose. Since the ability of palmitate to potentiate GSIS is partly dependent on its capacity to bind to GPR40, thereby increasing intracellular Ca^{2+} (239), we looked at the expression of GPR40 in 832/13 cells treated with siARNT. We found that the expression of GPR40 was reduced by 40 % in the absence of ARNT/HIF-1β (Figure 19 B). Thus, ARNT/HIF-1β seems to be necessary for the acute stimulation of GSIS by palmitate in 832/13 cells.

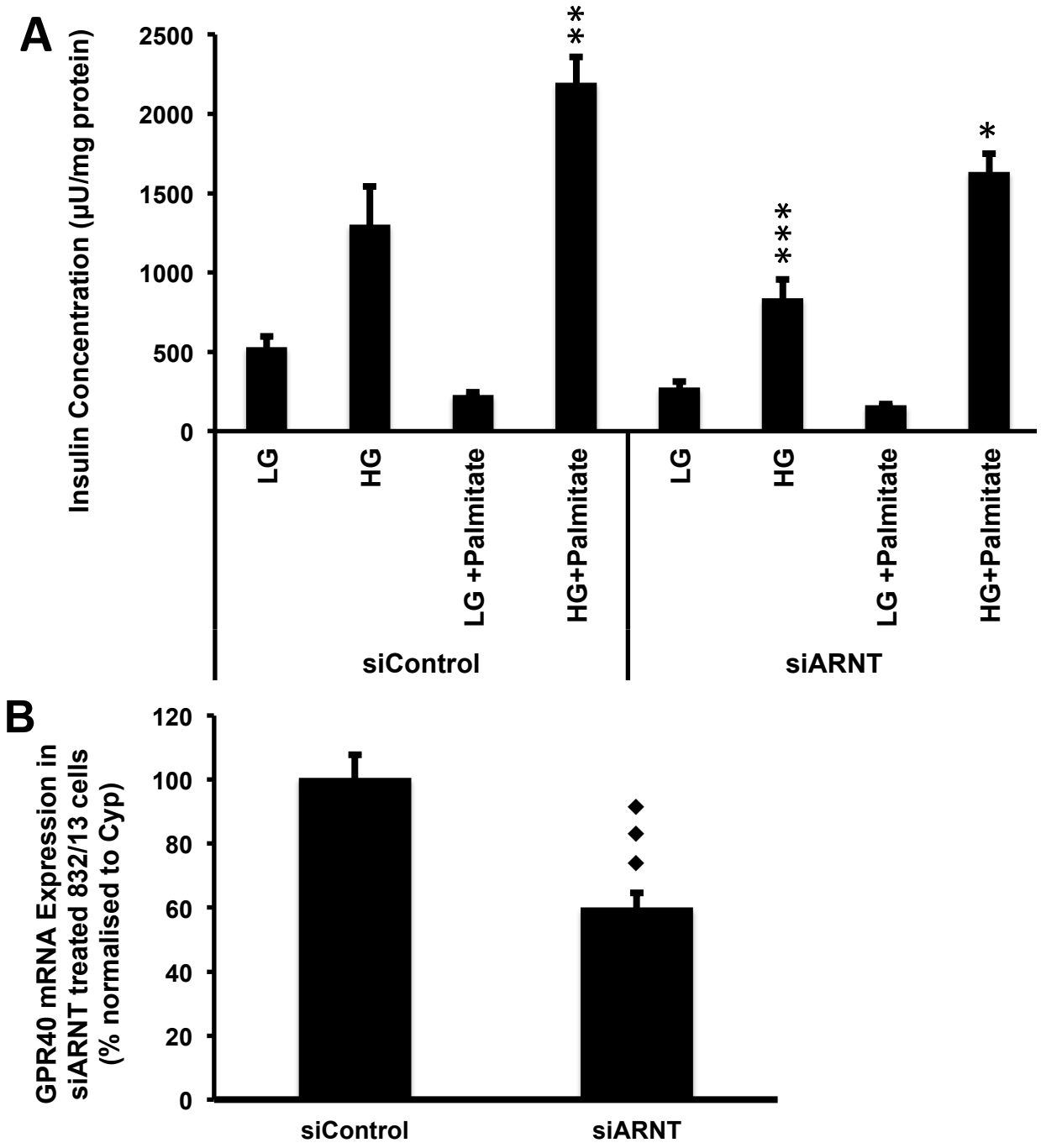

Figure 19: Fatty-acid potentiation of insulin secretion in siARNT-treated 832/13 cells. A) Insulin secretion in response to low glucose (LG, 2mM) and high glucose (HG, 7 mM) in the presence of palmitate (Pal, 250 µM). 2) mRNA expression of GPR40 in siARNT-treated cell. Data represents mean ± SEM. of 4 independent experiments. **p < 0.05 siControl HG + Pal Vs siARNT HG + Pal, **p<0.01 siControl HG Vs siControl HG+Pal, ***p<0.001 siControl HG Vs siARNT HG, ◆◆◆ p<0.001 siControl Vs siARNT. Student's t-test.*

3.5 Discussion

Genome-wide gene expression profiling of islets obtained from normal and type 2 diabetic humans revealed that the expression level of transcription factor ARNT/HIF-1β was reduced by 90% under diabetic conditions (164). Down regulation of ARNT/HIF-1β in mouse islets was also associated with reduction in the expression of several of its target genes such as those involved in glycolysis, insulin signalling and those that are necessary to maintain normal beta-cell function. In this chapter, we assessed the metabolic profile of the 832/13-pancreatic beta-cell line with low ARNT/HIF-1β expression levels to provide more insight into the mechanism of ARNT/HIF-1β mediated GSIS. Specifically, our aim was to understand the identity of key metabolic pathways regulated by ARNT/HIF-1β and understand the mechanism by which it helps sustain normal GSIS from pancreatic beta-cells.

Using an siRNA against ARNT/HIF-1β mRNA (siARNT), we first demonstrated that knocking down ARNT/HIF-1β mRNA and protein inhibits GSIS in 832/13 cells. Since insulin secretion from pancreatic beta-cells requires the complete metabolism of glucose through glycolysis and the TCA cycle, we decided to study the impact of ARNT/HIF-1β knock down on glucose utilization (glycolysis) and glucose oxidation (mitochondrial glucose metabolism) in 832/13 cells treated with siARNT. Although glucose utilization was decreased by 31% in siARNT cells, this reduction in glycolytic flux did not translate into changes in the rate of glucose oxidation or glucose - stimulated ATP production. To gain further insight into the metabolic phenotype of beta-cells deficient in ARNT/HIF-1β, GC-MS based metabolite profiling was carried out in 832/13 cells treated with siARNT. Consistent with ARNT/HIF-1β's known role as a master regulator of several key glycolytic enzymes such as phosphofructokinase (PFK), aldolase and glucose 6 phosphoisomerase (G6PI) (161,164), we found that glycolytic intermediates were significantly reduced in siARNT-treated cells; however, the most dramatic reductions were seen for metabolites involved in the TCA cycle suggesting that anaplerosis is an important target of ARNT/HIF-1β. These studies demonstrate that the role of ARNT/HIF-1β in regulating insulin release in response to glucose is independent of changes in glucose oxidation and ATP production and likely involves altered anaplerosis.

The critical involvement of glucose-stimulated anaplerosis in insulin secretion was confirmed by several studies (72,240-244). When glucose is abundantly present in the plasma, it enters the beta-cells where the metabolism of glucose into pyruvate prepares the hexose molecule to enter the mitochondria for further oxidation. The entry of pyruvate into the mitochondrial TCA cycle occurs via 2 distinct pathways, namely PDH mediated oxidative pathway and PC mediated anaplerotic pathway. In beta-cells, since PC is extremely active and highly expressed, PC catalyzed anaplerosis happens in almost equal proportions to the PDH catalyzed oxidation, giving rise to the existence of two separate pyruvate pools, with one feeding acetyl-CoA into the TCA cycle for oxidation and generation of ATP (PDH pathway) and the other leading to a net accumulation of TCA intermediates (PC pathway) (79). The novel finding that loss of ARNT/HIF-1β leads to a profound reduction in the expression of PC, DIC and OGC and a reduction in TCA metabolites, even though glucose oxidation and ATP production were unaltered, is an unexpected outcome of this study. It seems that the beta-cells in the absence of ARNT/HIF-1β preserve the ability to maintain their oxidative capacity in a bid to meet basic cellular energy demands and they do so at the expense of the anaplerotic input into the TCA cycle, leading to the defective secretion of insulin in response to glucose.

Loss of anaplerosis in beta-cells can have a significant impact on the 3 main pyruvate cycling pathways that are prevalent in the beta-cells, namely the pyruvate-malate, pyruvate-citrate and pyruvate-isocitrate pathways (79,88,91,225,226) Studies have shown that the flux through the 3 pyruvate cycling pathways strongly correlate with GSIS (70,78,240). The fact that there is a 50-70% reduction in the amounts of pyruvate, malate, citrate/isocitrate and α-ketoglutarate in siARNT-treated 832/13 cells suggests the possibility that the flux through all the 3 pyruvate cycling pathways may be compromised in the absence of ARNT/HIF-1β. It is also interesting to note that pyruvate cycling generates two potentially important signalling molecules for insulin release, namely, α-ketoglutarate and NADPH. NADPH can be produced via the three pyruvate cycling pathways (88,91,225). A key NADPH-producing enzyme for both the pyruvate malate and pyruvate citrate pathways is the cytosolic malic enzyme (MEc) (245). We found that MEc, which catalyzes the conversion of malate back to

pyruvate in the cytosol of the beta-cells, was significantly reduced in the absence of ARNT/HIF-1β. The key enzyme for the pyruvate isocitrate pathway is the cytosolic isocitrate dehydrogenase (ICDc) (91). However, we did not find any change in the expression of ICDc in the absence of ARNT/HIF-1β. Although strong evidence for a role of the pyruvate-isocitrate pathways exists (79,91), studies have also shown a role for the other two pyruvate cycling pathways (87,226,246) and therefore, the identity of the pyruvate cycling pathway(s) that is most critical for insulin release is still debated.

The observation that there is a complete lack of glucose induced rise in the production of fatty acids also supports the idea that anaplerosis is down regulated in siARNT-treated cells. In the normal scenario, when glucose is raised from basal to stimulatory concentrations, metabolism of glucose through glycolysis and TCA cycle leads to increased production of citrate. Citrate is then exported to the cytosol through the citrate/isocitrate carrier (CIC), where citrate then serves as a substrate for generation of lipid molecules such as malonyl CoA and LC-CoA, which have been suggested to serve as metabolic coupling factors in GSIS (65,66). Reduced levels of citrate in siARNT-treated cells and insufficient transport of citrate to cytosol may affect the ability of beta-cells to produce metabolic coupling factors necessary to maintain GSIS.

In an attempt to determine whether the secretory defects found in ARNT/HIF-1β deficient 832/13 cells are specific to glucose or affect other non-carbohydrate pathways as well, we examined insulin secretion in response to amino acids (leucine + glutamine), fatty acids (palmitate) and cAMP in 832/13 cells treated with siARNT. Leucine allosterically activates glutamate dehydrogenase and stimulates the conversion of glutamate to α-ketoglutarate, which can be oxidized in the TCA cycle to form ATP (247). It is also believed that leucine can be converted to ketoacid, α-ketoisocaproic acid (KIC), which can be then metabolized to hydroxymethyl glutarate –CoA (HMG-CoA) and acetyl CoA (227,248). Thus, two things can be inferred from an intact secretory response to leucine and glutamine in ARNT/HIF-1β deficient pancreatic beta-cells. 1) Oxidative capacity of beta-cells with reduced ARNT/HIF-1β is normal and the defects in insulin secretion seen are specific to glucose. 2) Since metabolism of leucine and glutamine generates ATP, potentially leading to the closure of the

K_{ATP} channels and insulin granule exocytosis, the K_{ATP} dependent pathway of insulin release is intact in the absence of ARNT/HIF1β. The potentiating effects of both palmitate and cAMP also seems to be intact in siARNT-treated 832/13 cells which again confirms the idea that defects in insulin secretion evident in ARNT/HIF-1β deficient 832/13 cells are specific to glucose.

In conclusion, based on our metabolomics and gene expression data, we strongly identify glucose-stimulated anaplerosis and pyruvate cycling as the two critical targets of ARNT/HIF-1β in pancreatic beta-cells. We show that ARNT/HIF-1β plays an important role in beta-cell glucose metabolism as siRNA-mediated knockdown of the transcription factor causes a significant reduction in gene expression, glycolytic flux and glucose-stimulated anaplerosis. Considering glucose oxidation and ATP production is intact in the absence of ARNT/HIF-1β, we believe that the observed metabolic phenotype can be attributed to a significant reduction in glucose-stimulated anaplerosis. The idea that low levels of ARNT/HIF-1β negatively affects the anaplerotic input into the TCA cycle is furthermore supported by the observation that glucose-induced fatty acid and glutamate production are completely absent in siARNT-treated 832/13 cells. Taken together, our data suggests that ARNT/HIF-1β is absolutely necessary to keep the pancreatic beta-cells in a glucose-responsive state and it does so by ensuring sufficient substrate flow through anaplerotic pathways that regulate GSIS.

Chapter 4

ARNT/HIF-1β is Indispensible for Maintaining Beta-Cell Secretory Function, but not for Glucose Homeostasis in Mice

4.1 Synopsis

ARNT/HIF-1β is a transcription factor that was previously shown to be critical for maintaining glucose homeostasis and glucose-stimulated insulin secretion (GSIS) in mice. Our goal was to understand the key metabolic signals regulated by ARNT/HIF-1β and evaluate its role *in vivo* in maintaining glucose homeostasis. Pancreatic beta-cell specific ARNT/HIF-1β knock-out mice (β-ARNT KO) and an ARNT/HIF-1β siRNA-treated 832/13 beta-cell line (siARNT) were used in this study. Both β-ARNT KO islets and siARNT cells showed impaired GSIS as expected. Mitochondrial oxygen consumption and mitochondrial membrane potential was unaltered upon glucose stimulation in β-ARNT KO islets. Further analysis of β-ARNT KO islets revealed impaired intracellular calcium response after stimulation with glucose and KCl. Subsequent analysis revealed reduced secretory response to glucose in the presence of KCl and diazoxide indicating a defect in the amplifying pathway of GSIS in β-ARNT KO islets. PC expression and the rise in glucose-stimulated NADPH/NADP ratio were completely absent in the absence of ARNT/HIF-1β. Consistent with this, the defect in GSIS in β-ARNT KO islets could be almost completely rescued by treatment with membrane permeable TCA intermediates. Surprisingly, β-ARNT KO mice have normal glucose homeostasis in both males and females. Further investigation revealed β-ARNT KO mice exhibited a small but significant increase in RER suggesting a preference in utilizing carbohydrates as a fuel source, possibly leading to improved glucose uptake from the blood stream. Response to exogenous insulin was completely normal in β-ARNT KO mice suggesting intact functioning of the skeletal muscles. Our data suggests an important role for ARNT/HIF-1β in maintaining normal beta-cell secretory function, however β-ARNT KO mice are protected from the adverse effects of hyperglycemia by activation of a compensatory mechanism in the peripheral tissues, the nature of which is currently unknown.

4.2 Introduction

Transcription factor ARNT/HIF-1β is important for a wide range of cellular functions such as response to hypoxia, angiogenesis, placental development and metabolism of xenobiotics (150). Although its role in the pathogenesis of type 2 diabetes (T2D) was previously unknown, Gunton et al's discovery in 2005, recognized ARNT/HIF-1β as one of the most critical transcription factors required to maintain normal beta-cell function. The discovery that ARNT/HIF-1β was 90% down-regulated in human type 2 diabetic islets and that it was necessary to maintain normal glucose homeostasis and insulin secretion in mice, provided a novel and unexpected role for this transcription factor in the beta-cells (164). The importance of ARNT/HIF-1β in GSIS, beta-cell function and in the progression of T2D was further supported by several recent *in vivo* studies performed by different research groups. Cheng et al showed that increasing the expression of ARNT/HIF-1β and its binding partner HIF-1α in human T2D islets markedly improved GSIS (170). The involvement of ARNT/HIF-1β in the pathogenesis of T2D was also evident in a few studies where ARNT/HIF-1β was found to be down-regulated in other metabolic tissues of the body such as the liver and the adipose tissue in humans and mice respectively (173,182,210). Ablation of ARNT/HIF-1β in liver was associated with glucose intolerance in mice. In adipose tissue however, the functional significance of ARNT/HIF-1β seems to be more complex than in beta-cells. Studies done by Jiang et al suggests that impaired HIF-1 signalling in the adipose tissue protects these mice from the deleterious effects of a high fat diet, leading to normal glucose homeostasis, improved insulin sensitivity and energy regulation. Improved insulin sensitivity in these mice was attributed to the decreased expression of Soc3 in the white adipose tissue and induction of adiponectin (183). However, in another study where the expression levels of HIF-1α, ARNT/HIF-1β binding partner, was inhibited by the expression of a dominant negative form of the protein, mice were found to be glucose intolerant and insulin resistant compared to wild-type littermates (184). Thus, several *in vivo* as well as *in vitro* data have been presented to the scientific community since 2005 providing compelling evidence pointing to the involvement of ARNT/HIF-1β in the progression and pathogenesis of T2D.

In the previous chapter, we did a detailed analysis of the metabolic phenotype of INS-1 derived 832/13 clonal beta-cells with low levels of ARNT/HIF-1β which revealed a reduction in glucose utilization, without affecting glucose oxidation and ATP/ADP ratio (249). Metabolic profiling of 832/13 cells deficient in ARNT/HIF-1β demonstrated reduced levels of glycolytic end product pyruvate and TCA intermediates citrate/isocitrate, α-ketoglutarate, fumarate and malate suggesting impaired anaplerosis. Taken together, we along with others have shown that ARNT/HIF-1β is undoubtedly one of the key transcription factors in pancreatic beta-cells, critical for maintaining GSIS, glucose-stimulated anaplerosis and glucose homeostasis in mice.

In this study, beta-cell specific ARNT/HIF-1β knock-out mice (β-ARNT KO) were generated using the Cre-loxP recombination system, where the Cre transgene was under the control of rat insulin promoter (RIP-Cre). The main goal of this study was to determine the role of ARNT/HIF-1β in maintaining glucose homeostasis *in vivo*. Gunton et al (164) had presented some valuable data on the *in vivo* role of ARNT/HIF-1β in mice where they found that the β-ARNT KO mice were glucose intolerant, with impaired insulin secretion and altered islet gene expression. However, they did not perform a detailed analysis of the metabolic phenotype of islets from β-ARNT KO mice. Specifically, our aim was three fold:
1) Functionally characterize islets from β-ARNT KO mice 2) identify the metabolic signals that are critically regulated by ARNT/HIF-1β for maintaining normal GSIS in mice 3) identify the mechanism by which ARNT/HIF-1β maintains glucose homeostasis *in vivo*.

As previously mentioned in the experimental procedures (section 2.14), generation of a beta-cell specific knock-out mice requires two types of transgenic mice: mice that express Cre recombinase in a tissue-specific manner (RIP-Cre) and mice that have the gene of interest flanked by loxP sequence (ARNT $^{Fl/Fl}$). Recently it has been shown that presence of the RIP-Cre transgene alone leads to glucose intolerance in mice (250). To overcome any variation in phenotype due to the presence of RIP-Cre and to establish that the phenotype observed in β-

ARNT KO mice is due to the deletion of ARNT/HIF-1β protein, all controls used for *in vivo* studies were RIP-Cre positive (WT, Cre or WT/Fl, Cre).

Using β-ARNT KO mice and 832/13 cells treated with siRNA against ARNT mRNA (siARNT), we show that ARNT/HIF-1β plays a critical role in maintaining the glucose-stimulated rise in NADPH/NADP ratio and rise in intracellular Ca^{2+} levels, two major stimulus-secretion coupling factors required to maintain normal GSIS from the pancreatic beta-cells. However, contrary to previous reports, we found that both male and female β-ARNT KO mice have normal glucose homeostasis and these mice exhibited increased utilization of carbohydrates as a fuel source, possibly protecting them from the adverse effects of hyperglycemia.

4.3 Contributions

Evaluation of whole body bioenergetics in mice using CLAMS was carried out in collaboration with Dr. Russell Tupling and Dr. Eric Bombardier, Department of Kinesiology, University of Waterloo. Calcium and mitochondrial membrane potential measurements were carried out in collaboration with Dr. Mike Wheeler and Kacey Prentice, Department of Physiology, University of Toronto, Toronto, ON, Canada.

4.4 Results

4.4.1 Genotyping

To study the role of ARNT/HIF-1β in pancreatic beta-cells, we employed Cre-loxP technology as previously described in the experimental procedures to specifically delete the gene in pancreatic beta-cells. RIP-Cre mediated recombination resulted in the deletion of exon 6 that encodes the bHLH region of the ARNT/HIF-1β protein. As expected, a PCR based genotyping for the detection of loxP targeted ARNT-floxed allele and the RIP-Cre gene resulted in a 290 bp fragment for the wild-type allele, a 340 bp fragment for the homozygous ARNT-floxed allele and a 553 bp for the RIP-Cre transgene (Figure 20 A, B, C)

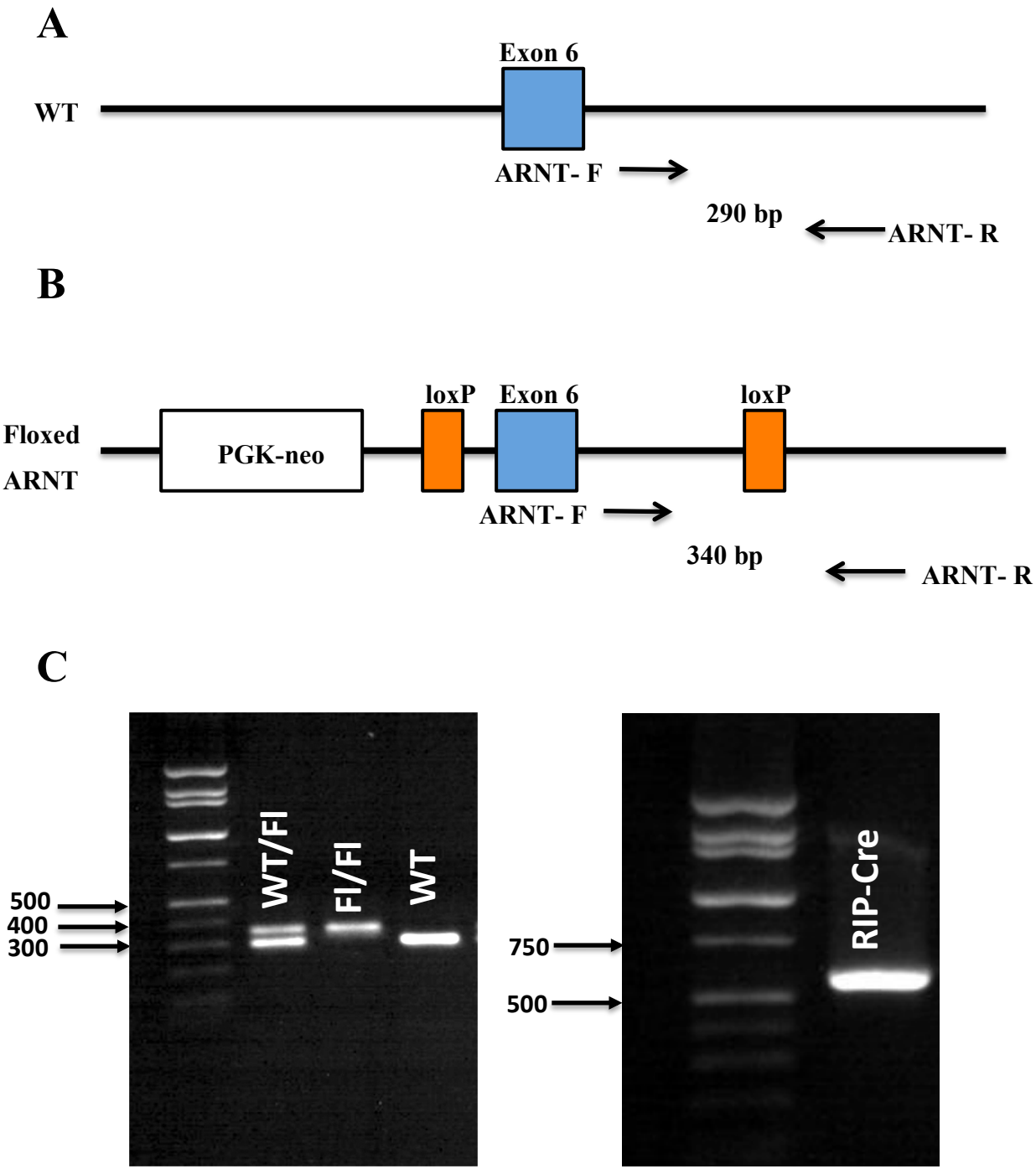

Figure 20: Schematics of PCR based genotyping for the detection of A) wild-type allele (WT) B) ARNT floxed allele. C) Representative PCR genotyping for ARNT-floxed allele and Cre recombinase transgene obtained using mouse tail genomic DNA. PCR products were separated on a 1 % agarose gel and visualized by ethidium bromide staining. A100-bp DNA ladder was used as a size marker for both ARNT-floxed and RIP-Cre genes. Black arrows in A, B represent the position of the forward (ARNT-F) and reverse (ARNT-R) primers used for genotyping.

4.4.2 Effective Deletion of ARNT/HIF-1β mRNA in β-ARNT KO islets and INS-1 832/13 cell-line

A quantitative PCR analysis of the expression of ARNT/HIF-1β mRNA revealed 76.8% and 60.6% reduction in gene expression in β-ARNT KO islets compared to WT,Cre and WT/Fl,Cre control mice islets respectively. ARNT mRNA expression was not found to be significantly different between WT,Cre and WT/Fl, Cre mice islets used in this study (Figure 21 A). ARNT/HIF-1β was also knocked down using an siRNA in the 832/13 beta-cell line (validated in the first chapter), which resulted in an efficient 76% knockdown of ARNT/HIF-1β mRNA (Figure 21 B).

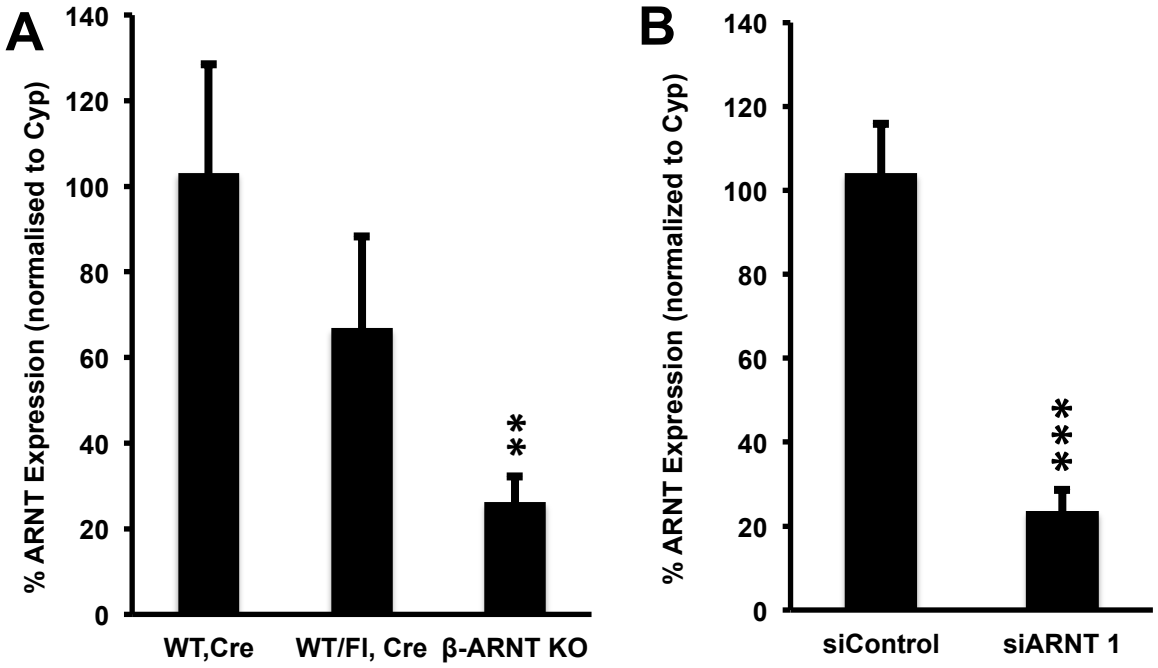

Figure 21: Deletion of ARNT/HIF-1β in β-ARNT KO mice and 832/13 cells A) qPCR analysis of ARNT/HIF-1β mRNA in islets from wild-type and positive for Cre (WT,Cre), heterozygous ARNT floxed and positive for Cre (WT/Fl, Cre) and β-ARNT KO mice. B) qPCR analysis of ARNT/HIF-1β mRNA in 832/13 cells treated with siARNT compared to siControl-treated cells. Results represent mean ± SEM 3 – 5 mice for each genotype and 3 – 5 independent experiments run in duplicates for siARNT 832/13 cells. *p<0.05 WT/Fl, Cre Vs *β-ARNT KO islets,** p<0.01 for WT, Cre Vs β-ARNT KO islets, *** p<0.001 for siControl Vs siARNT*.

4.4.3 Fasting Blood Glucose Measurements

Blood glucose measurements were taken in both male and female β-ARNT KO mice after 12 h of fasting. Blood samples were collected from the tail clipping and glucose concentration was measured using a glucose meter. We did not observe any difference in the glucose values between β-ARNT KO and control mice (WT,Cre and WT/Fl,Cre) in both males and females (Figure 22).

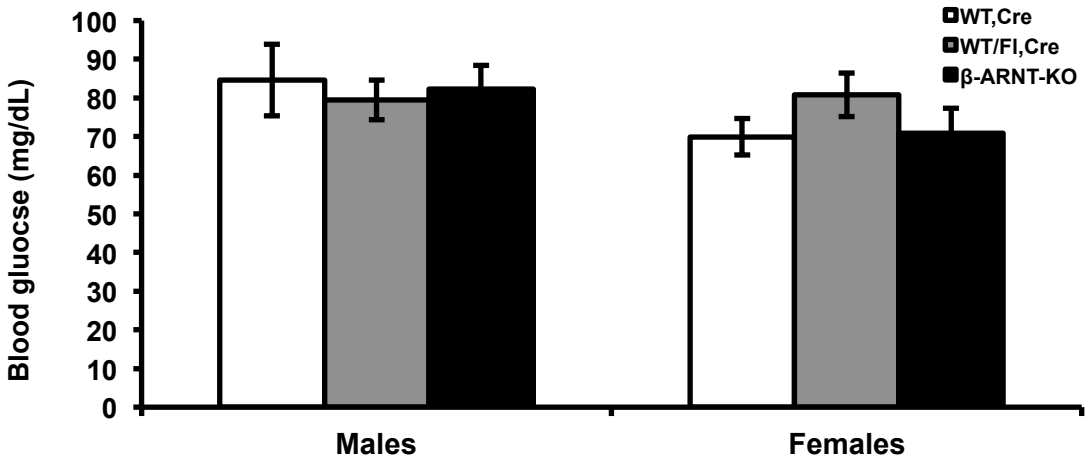

Figure 22: Fasting blood glucose measurements in male and female β-ARNT KO mice. Following 12 hours of fasting, blood samples were collected from the tail vein and glucose was measured using a glucose meter. n=6 -10 per genotype for both males and females. Results represent mean ± SEM.

4.4.4 Intraperitoneal Glucose Tolerance Test (ipGTT) in β-ARNT KO mice

An ipGTT was performed on 15-20 week old mice after 12-16 h of fasting with both male and female β-ARNT KO mice to determine whether disruption of ARNT/HIF-1β had an impact on glucose homeostasis. Interestingly and contrary to previous reports, we did not find any difference after an intraperitoneal injection of 1.5g/kg body weight glucose in both male and female β-ARNT KO mice compared to their control littermates (Figure 23 A, C). To further assess the overall glucose clearance during the entire span of the ipGTT, incremental area under the curve (AUC) was calculated, which also did not show any statistically significant difference between male and female β-ARNT KO mice compared to

their controls (Figure 23 B, D). Both male and female mice with beta-cell specific deletion of ARNT/HIF-1β were previously shown to be glucose intolerant (164).

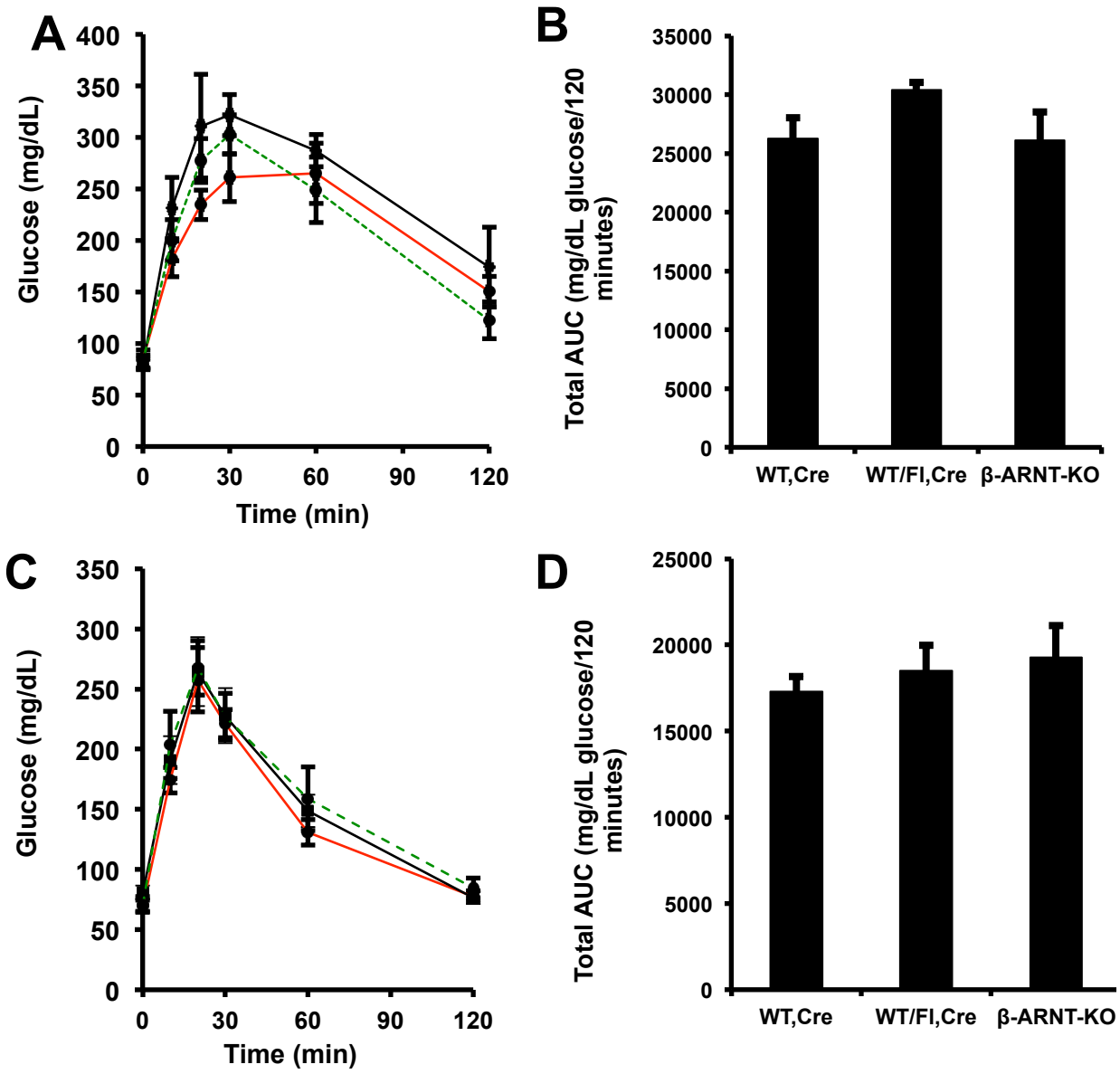

Figure 23: Intraperitoneal glucose tolerance test (ipGTT) in β-ARNT KO mice. A) ipGTT in male β-ARNT KO mice (green trace) in comparison with male ARNT WT/Fl, Cre controls (black trace) and WT, Cre controls (red trace) B) AUC for male β-ARNT KO mice C) ipGTT in female β-ARNT KO mice (green trace) in comparison with ARNT WT/Fl, Cre controls (black trace) and WT,Cre controls (red trace). D) AUC for female β-ARNT KO mice. n=6 -10 per genotype for both males and females. Results represent mean ± SEM.

4.4.5 Glucose-Stimulated Insulin Secretion in β-ARNT KO Islets

Our observation that both male and female β-ARNT KO mice had normal glucose homeostasis was quite unexpected and not in agreement with a previously published report by Gunton et al (164). To determine whether islets from β-ARNT KO mice responded normally to glucose and secreted insulin in the normal amounts, a glucose-stimulated insulin secretion (GSIS) assay was performed on islets from both male and female β-ARNT KO mice. At 2 mM glucose, we found hyper secretion of insulin in islets from male β-ARNT KO mice, where they secreted 52 % more compared to islets from control mice. Although islets from female β-ARNT KO mice exhibited a similar trend, results were not statistically significant. At 16.7 mM glucose, islets from male β-ARNT KO secreted ~ 46 % less insulin and islets from female β-ARNT KO mice secreted ~ 42 % less insulin compared to control islets (Figure 24). Thus, glucose-stimulated insulin secretion was found to be defective in islets from β-ARNT KO mice.

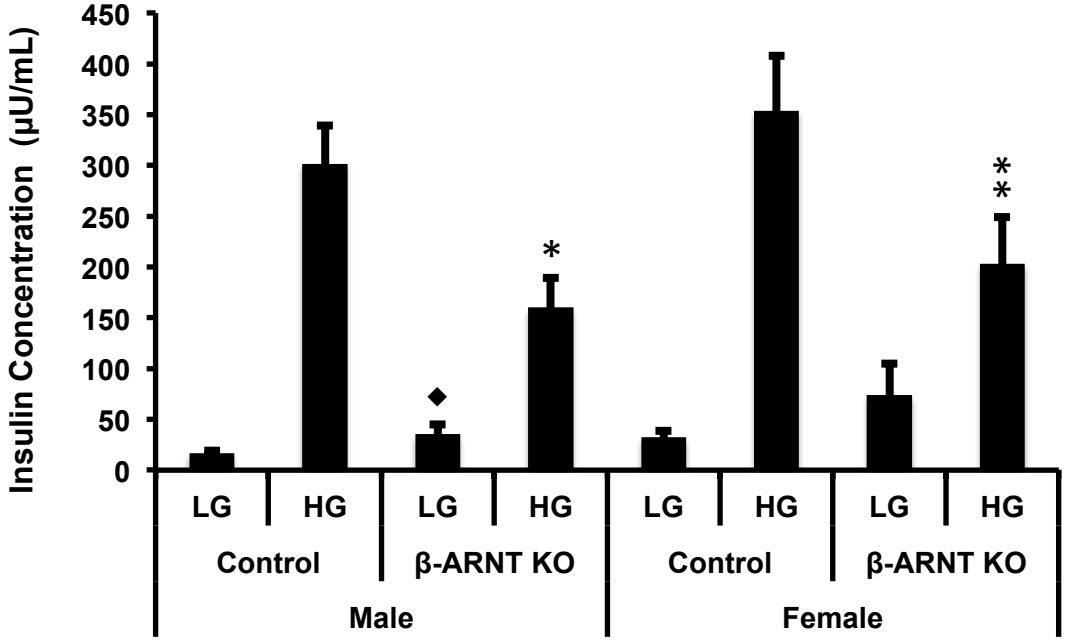

Figure 24: Glucose-stimulated insulin secretion in male and female β-ARNT KO islets. LG, low glucose (2 mM); HG, high glucose (16.7 mM). Results represent mean±SEM of 3-6 independent experiments. *◆p<0.05 LG male control islets Vs LG β-ARNT KO islets. *p<0.05 HG male control islets Vs HG β-ARNT KO islets; **P<0.01 HG female control islets Vs HG β-ARNT KO islets. Student's t-test.*

4.4.6 Evaluating Whole Body Bioenergetics in β-ARNT KO mice

In an attempt to understand the mechanism by which β-ARNT KO mice maintain normal glucose homeostasis despite a significant reduction in GSIS observed in β-ARNT KO islets, we decided to evaluate the whole body metabolic changes that are associated with beta-cell specific deletion of ARNT/HIF1β. Metabolic cages were used to assess food intake, locomotive activity (dual beam activity), volume of oxygen consumption (VO_2) and volume of carbon dioxide exhaled (VCO_2) in 15-20 week old male β-ARNT KO mice. Measurements were all normalized to body weight and the mice had *ad libitum* access to food and water. We did not find any significant differences in food intake, body weight and dual beam activity in β-ARNT KO mice compared to their control littermates (Figure 25 A, B, C). However, respiratory exchange ratio (RER), which is the ratio of VCO_2 /VO_2 was slightly but significantly higher in β-ARNT KO mice (0.99) compared to their control littermates (0.93) (Figure 25 D). RER closer to 1 signifies preferential utilization of carbohydrates and closer to 0.7 suggests utilization of fat for meeting the whole body energy demands (251). Increased RER in β-ARNT KO mice suggests that these mice may utilize more glucose compared to their controls, possibly leading to normal glucose homeostasis. Some studies have shown that RIP-Cre can also be expressed in the hypothalamus, often leading to the deletion of the floxed gene in the hypothalamus, negatively impacting food intake and body weight (250). The fact that neither food intake nor the body weight was affected implies that RIP-Cre mediated recombination events may not have had the expected impact in the hypothalamus of β-ARNT KO mice and that ARNT/HIF-1β function may not be compromised in the brain.

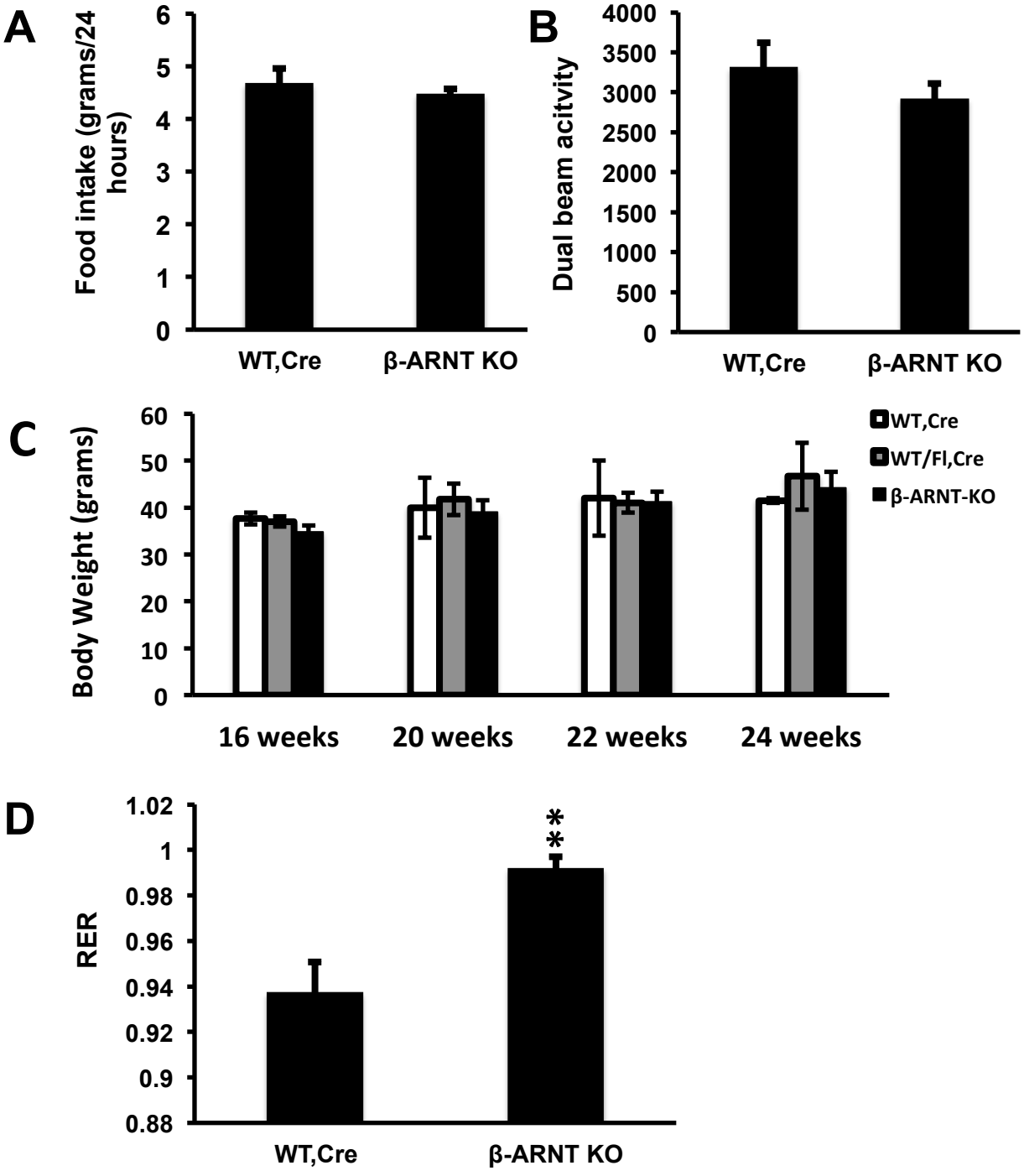

Figure 25: Indirect calorimetry measurement of whole body bioenergetics in male β-ARNT KO mice A) Food intake B) Dual beam activity C) Body weight D) RER. n=8 per genotype. Data represents mean ± SEM. ***p<0.01 WT,Cre Vs β-ARNT KO. Student's t-test.*

4.4.7 Insulin Tolerance Test (ITT) in β-ARNT KO mice

The fact that β-ARNT KO mice had a higher RER in comparison to control mice suggested the possibility of improved sensitivity of skeletal muscles to glucose thereby leading to better glucose disposal. In order to test this hypothesis, an intraperitoneal insulin tolerance test was conducted in both male and female β-ARNT KO mice. 1.5 IU/kg and 1.2 IU/kg of insulin were injected intraperitoneally in male and female mice respectively after 4 h fasting. In male β-ARNT KO mice, although overall insulin sensitivity was better, this was not statistically significant. However, we observed improved glucose disposal rate at several time points, which was found to be statistically significant at 60 min compared to the control mice. (Figure 26 A, B). In female β-ARNT KO mice, no significant differences were observed in insulin sensitivity between β-ARNT KO and control mice (Figure 26 C, D).

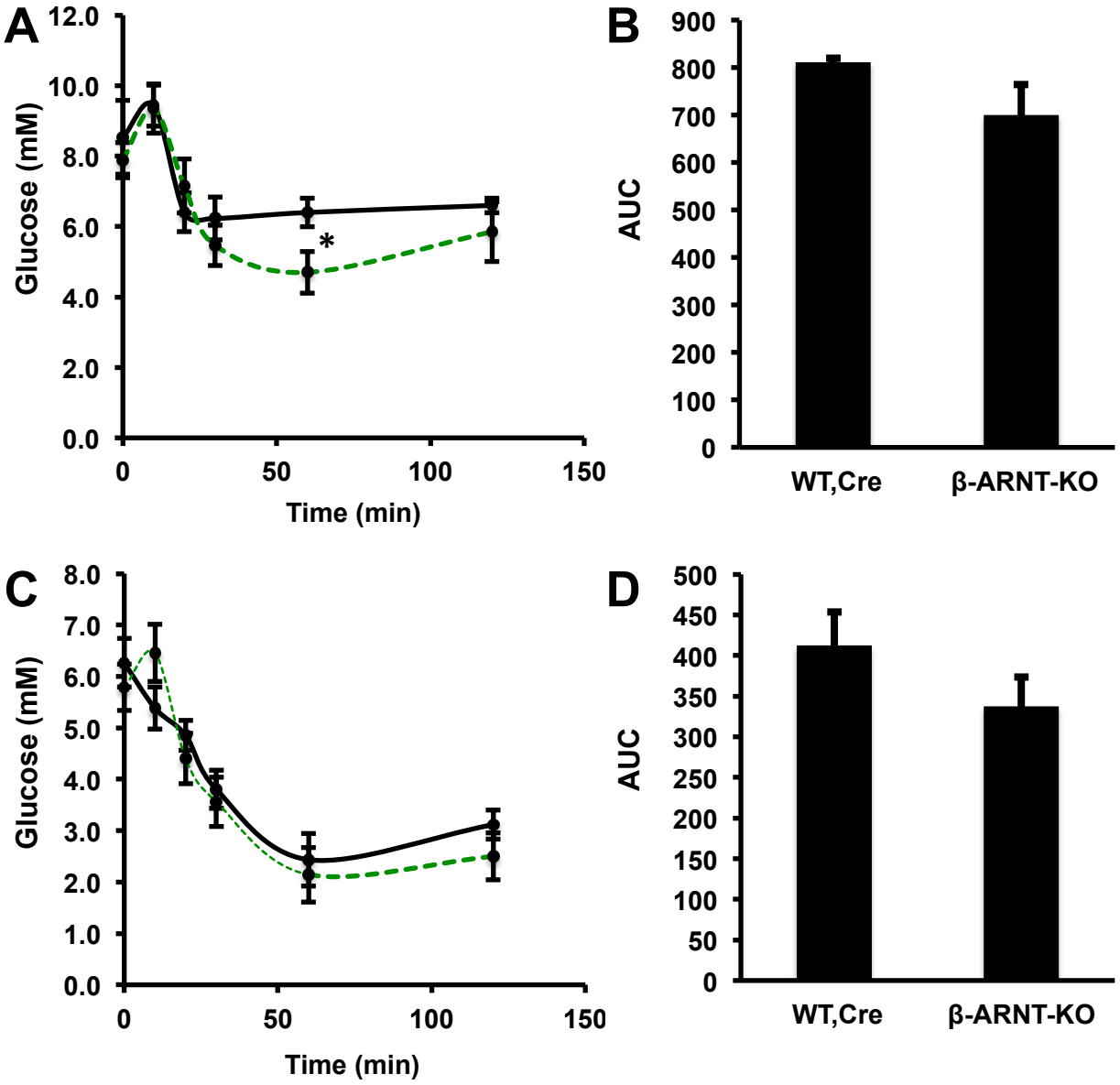

Figure 26: Insulin tolerance test (ITT) in male and female β-ARNT KO mice. A) ITT in male β-ARNT KO mice (green trace) compared to WT,Cre controls (black trace) B) Incremental area under the curve (AUC) to assess overall glucose clearance in male β-ARNT KO mice C) ITT in female β-ARNT KO mice (green trace) compared to WT,Cre controls (black trace). D) Incremental area under the curve (AUC) to assess overall glucose clearance in female β-ARNT KO mice. n= 6 – 7 per genotype for ITT and data represent mean ± SEM. **p<0.05 glucose clearance in WT,Cre Vs β-ARNT KO mice at 60 minutes. Student's t-test.*

4.4.8 Mitochondrial Oxygen Consumption Rate (OCR) in β-ARNT KO Islets

To further investigate mechanisms underlying altered insulin response in β-ARNT KO islets, we decided to examine the mitochondrial function by measuring the OCR using a Seahorse flux analyzer. Under stimulatory glucose concentration, the OCR ramps up in response to increased oxidative phosphorylation. In addition to glucose, we also looked at the OCR in response to various mitochondrial drugs to understand the mitochondrial bioenergetics in the absence of ARNT/HIF1β. As suggested by our glucose oxidation measurements from 832/13 cells with low levels of ARNT/HIF1β, we found that at both 2 mM and 16.7 mM glucose, OCR was not significantly different in β-ARNT KO islets compared to control islets (Figure 27 A). This suggests that the oxidative capacity was not reduced in these cells. OCR in response to mitochondrial drugs oligomycin (ATP synthase inhibitor), 2, 4 dinitrophenol (DNP - uncoupler) and rotenone and myxothiazol (electron transport chain inhibitor) were also not significantly different in β-ARNT KO islets compared to control islets. Mitochondrial function was also studied in 832/13 cells treated with siARNT. We found a very similar trend where the OCR in response to glucose, oligomycin, DNP and rotenone + myxothiazol in siARNT-treated cells was comparable to siControl cells (Figure 26 B).

In addition to measuring the OCR, the use of mitochondrial drugs also allows us to study various mitochondrial parameters such as the ATP turn over, proton leak and spare respiratory rate in our cells. The rate of mitochondrial ATP turn over was estimated by comparing the difference in OCR upon inhibiting ATP synthase to OCR in the presence of either 2 mM or 16.7 mM glucose. Proton leak is inversely related to mitochondrial efficiency and is estimated by comparing the difference in OCR upon inhibiting ATP synthase to OCR in the presence of respiratory chain inhibitors, rotenone and myxothiazol. Finally, spare respiratory capacity, which denotes the ability of cells to respond to an increase in energy demand, was calculated by measuring the difference in OCR after the injection of DNP compared to OCR at either 2 mM or 16.7 mM glucose. It also indicates how close the cells are operating to their bioenergetics limits (252). All parameters were found to be similar in siARNT-cells compared to siControl-cells (Figure 28 A, B, C). These results indicate that

loss of ARNT/HIF-1β does not lead to mitochondrial dysfunction and that this is not a mechanism through which the transcription factor regulates GSIS.

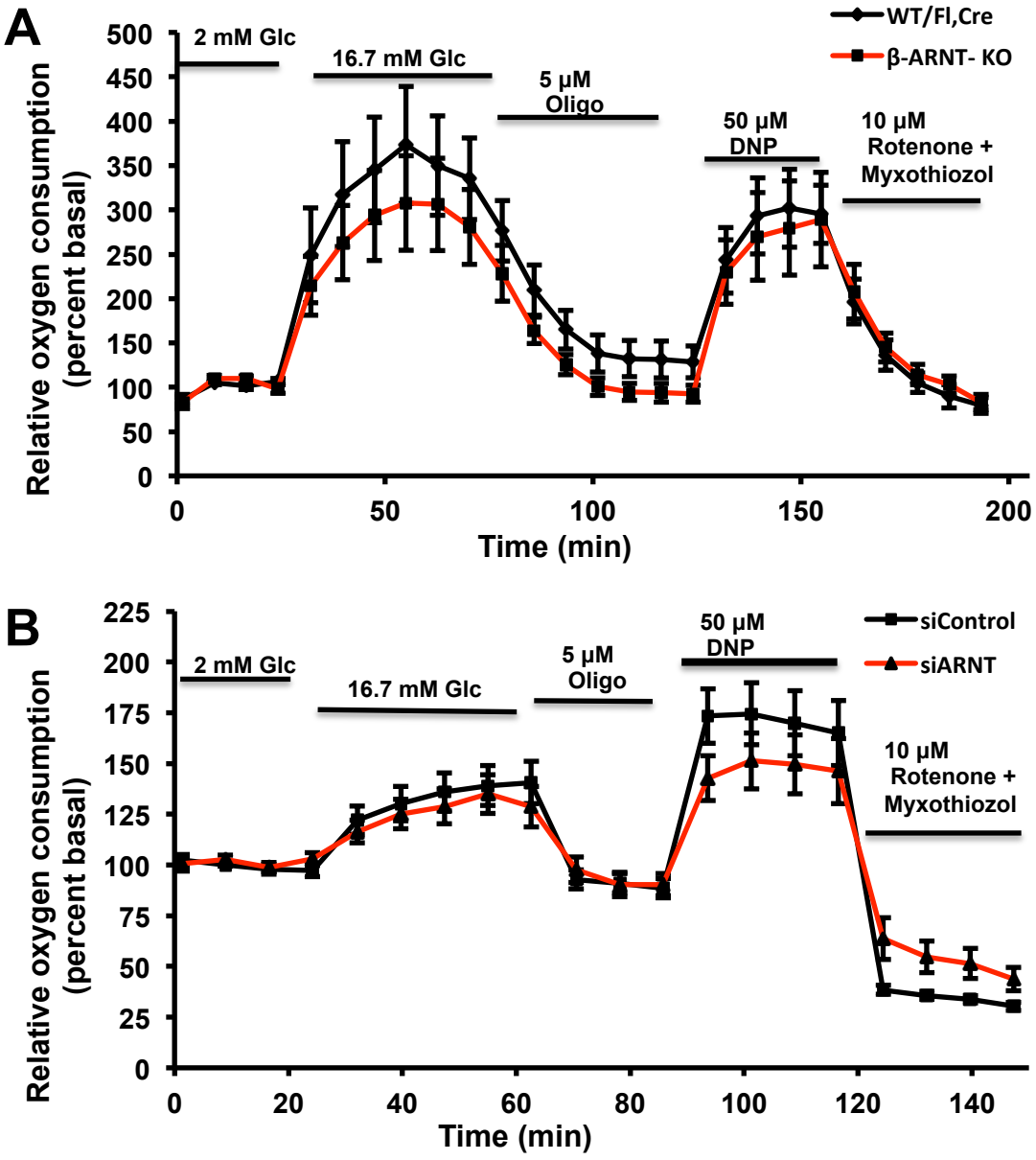

Figure 27: Mitochondrial oxygen consumption rate in A) Mice islet - WT/Fl, Cre (black trace) compared to β-ARNT KO islets (red trace) B) siARNT treated 832/13 cells**.** siControl cells (black trace) compared to siARNT-treated 832/13 cells (red trace) under basal 2 mM glucose (Glc), 16.7 mM glucose (glc), 5 µM oligomycin (Oligo), 50 µM di-nitrophenol (DNP) and 10 µM each of rotenone and myxothiazol. Results represents mean ± SEM 3 – 4 independent experiments (3 mice per genotype and ~ 50-60 islets per mice). For siRNA treatment, n=4 independent experiments with a minimum of 4 replicates per experiment.

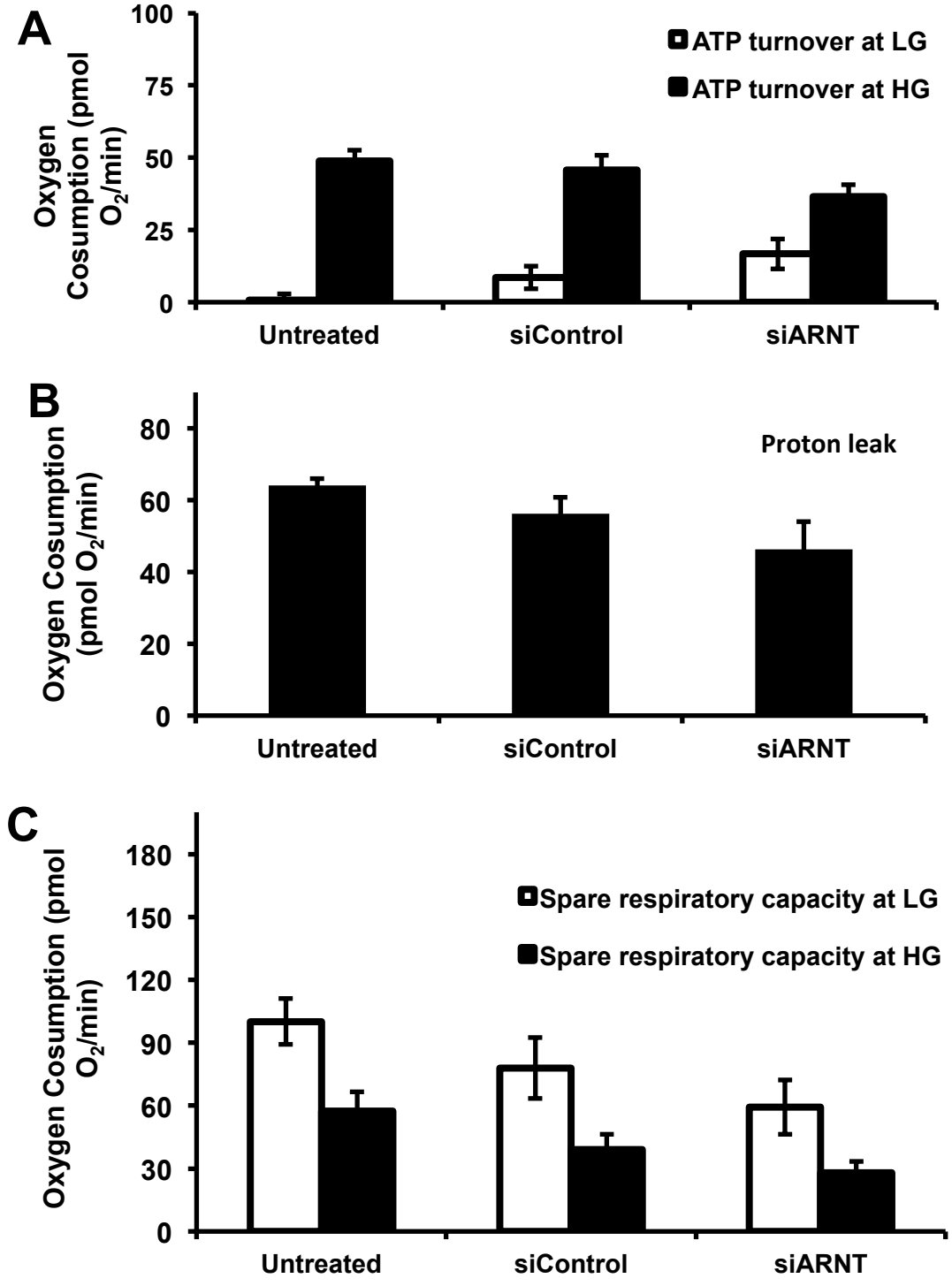

Figure 28: Evaluating mitochondrial bioenergetics in 832/13 cells treated with siARNT. A) ATP turn over at 2 mM (LG) and 16.7 mM (HG) glucose B) Proton leak C) Spare respiratory capacity at 2 mM (LG) and 16.7 mM (HG) glucose. Results represent mean ± SEM 3 – 4 independent experiments.

4.4.9 Mitochondrial Membrane Potential (MMP) in β-ARNT KO Islets

Upon glucose stimulation, an increase in proton motive force inside the mitochondria leads to hyperpolarization of the inner mitochondrial membrane and significant increase in ATP synthesis. To investigate the mechanisms underlying defective insulin secretion observed in β-ARNT KO islets, MMP was measured upon glucose stimulation using the mitochondrial specific dye rhodamine 123 (Rh123). A representative trace shows that addition of 20 mM glucose results in the hyperpolarization of the mitochondrial membrane and addition of 1 mM sodium azide (NaN_3) a respiratory chain inhibitor, results in the depolarization of mitochondrial membrane potential in control islets. As expected, β-ARNT KO islets did not show any difference in MMP upon stimulation with 20 mM glucose when normalized to baseline (Figure 29). As changes in MMP often indicate perturbations in ATP production, our data suggest that the process of oxidative phosphorylation and ATP production is undisturbed in β-ARNT KO islets.

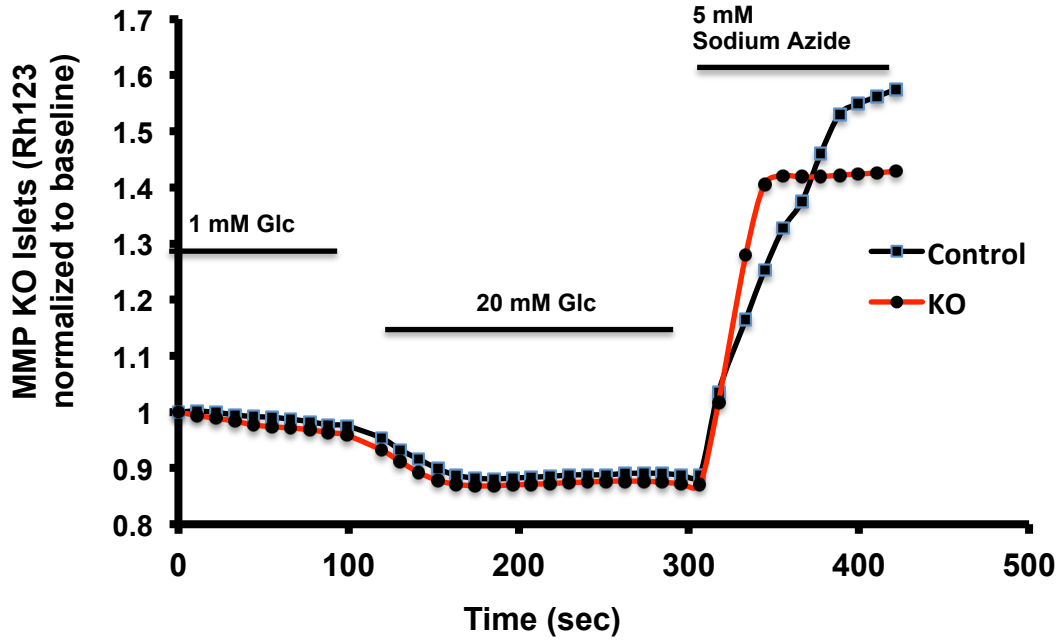

Figure 29: Mitochondrial membrane potential in β-ARNT KO islets. Representative traces of Rhodamine 123 (Rh123) fluorescent signal in β-ARNT KO islets (red trace) compared to control islets (black trace). No significant differences were observed between β-ARNT KO and control islets. (n=4 mice per group, with a minimum of 50 islets per mice).

4.4.10 Defects in Anaplerosis and Amplifying Pathway of GSIS in β-ARNT KO Islets

Lack of impairment in mitochondrial membrane potential and cellular oxygen consumption rates in β-ARNT KO islets suggest that ARNT/HIF-1β regulates GSIS independent of the triggering or the K_{ATP} dependent pathway of insulin release and that defective insulin secretion could be attributed to perturbations in the amplifying pathway of GSIS. Since our previous results from 832/13 cells with low ARNT/HIF-1β expression also suggested the same, we decided to investigate whether the amplifying pathway is operational in β-ARNT KO islets. Insulin secretion was assessed in β-ARNT KO islets in the presence of KCl (30 mM) and diazoxide (200 μM) at both 2 mM and 12 mM glucose (Figure 30 A). Diazoxide keeps the K_{ATP} channels open and KCl depolarizes the beta-cell membrane directly and clamps the cytoplasmic Ca^2 at high levels, thus bypassing the K_{ATP} channel function and the triggering pathway of GSIS. At 2 mM glucose, β-ARNT KO islets secreted 76 % less insulin compared to control islets. Since depolarizing the membrane with 30 mM KCl in the presence of diazoxide and 2 mM glucose, increases intracellular Ca^{2+}, loss of insulin secretion in β-ARNT KO islets under these conditions, indicate possible defects in the Ca^{2+} influx. At 12 mM glucose, β-ARNT KO islets secreted 19 % less insulin compared to control islets indicating a key role for ARNT/HIF-1β in the metabolic amplifying pathway of GSIS.

Anaplerosis and signals generated from glucose-stimulated anaplerosis play critical role in the amplifying pathway of GSIS. Since pyruvate carboxylase (PC) is the master regulator of anaplerosis that modulates the flow of substrates through the anaplerotic pathway of the TCA cycle, we measured the expression of PC in β-ARNT KO islets. Loss of ARNT/HIF-1β leads to a 57% reduction in the expression of PC in pancreatic islets (Figure 30 B). Down-regulation of PC will likely lead to a decrease in the substrate flux through the TCA cycle as shown previously in siARNT-treated 832/13 cells (249), thus having an impact on the generation of anaplerotic signals necessary for GSIS.

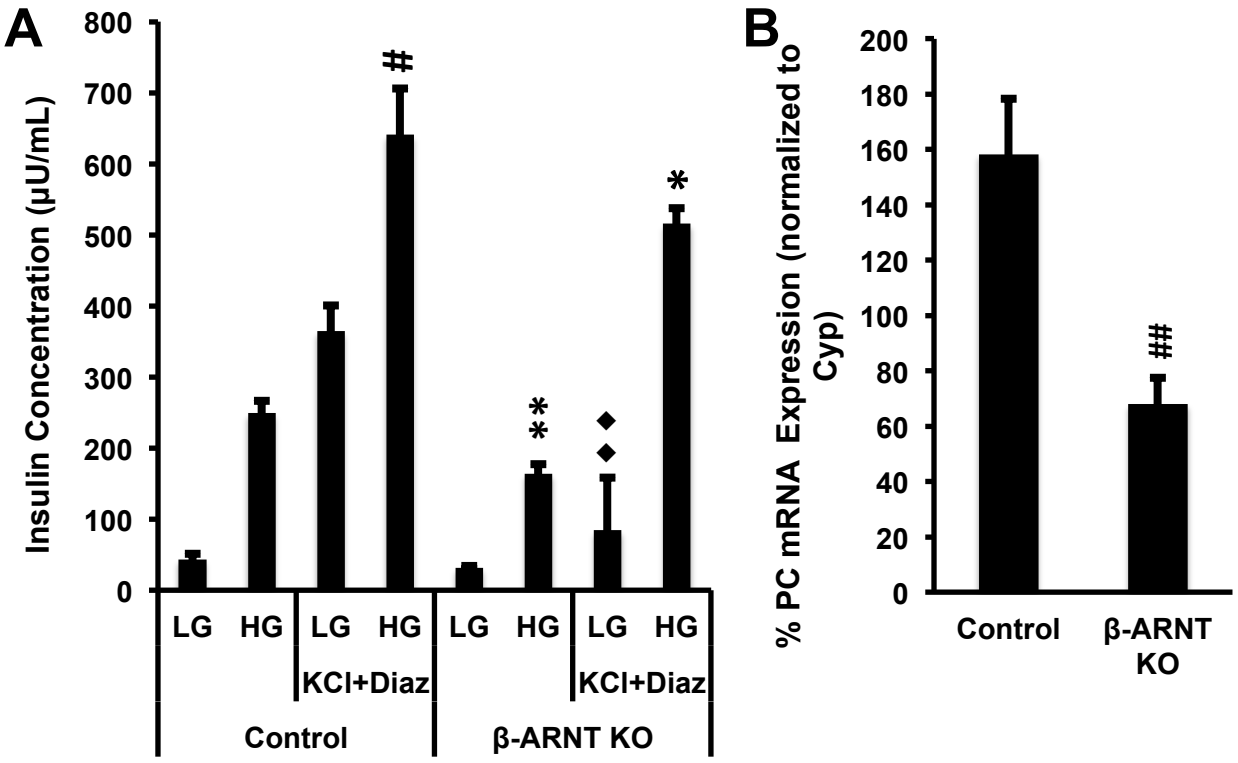

Figure 30: Involvement of amplifying pathway and anaplerosis in ARNT/HIF-1β mediated GSIS A) GSIS in β-ARNT KO islets in the presence of 30 mM KCl and 200 µM Diazoxide (Diaz) with either 2 mM (LG) or 12 mM (HG) glucose B) qPCR analysis of pyruvate carboxylase (PC) expression in β-ARNT KO. Results represent mean ± SEM of 3- 4 independent experiments. ** p<0.05 HG Control KCl+Diaz Vs HG β-ARNT KO KCl+Diaz. ** p<0.01 HG Control vs. HG β-ARNT KO islets, ◆◆ p<0.01 LG Control KCl+Diaz vs. LG β-ARNT KO KCl+Diaz, #p<0.05 Control HG Vs Control HG+KCl, ## p<0.01 PC expression in Control vs. β-ARNT KO islets. Student's t-test.*

4.4.11 Rescue of Insulin Secretion by Dimethyl Alpha Ketoglutarate (DMAKG) and Dimethyl Malate (DMM)

Our metabolomics data from 832/13 cells treated with siARNT indicated that anaplerosis is severely blunted in the absence of ARNT/HIF1β. If impaired GSIS in β-ARNT KO islets is due to loss of glucose-stimulated anaplerosis as in 832/13 cells with low ARNT/HIF-1β levels, then replenishing anaplerotic substrates should rescue GSIS in β-ARNT KO islets. In order to test this hypothesis, we treated β-ARNT KO islets with a combination of 5 mM each

of DMAKG and DMM, membrane permeable esters of alpha ketoglutarate and malate and assessed insulin secretion in the presence of 12 mM glucose. Both DMAKG and DMM were shown to increase insulin secretion in the presence of glucose through stimulation of the anaplerotic pathway (70,77,245). As shown in Figure 31A, in the presence of 12 mM glucose, insulin secretion was decreased by around 30% in β-ARNT KO islets. However, the addition of 5 mM each of DMAKG and DMM nearly doubled GSIS in both control and β-ARNT KO islets.

A similar rescue experiment was also carried out in 832/13 cells treated with siARNT. In these clonal cells, a glucose concentration of 7 mM was found to be optimal for the potentiating action of DMAKG and DMM. Both siControl and siARNT cells were treated with 5 mM each of DMAKG and DMM in the presence of 7 mM glucose. A shown in Figure 31 B, siARNT-treatment resulted in almost 50 % reduction in GSIS compared to siControl cells. However, GSIS was rescued to almost near-normal levels by the addition of DMAKG and DMM along with 7 mM glucose. These results show that GSIS can be rescued in β-ARNT KO islets if the flow of substrates through the TCA cycle is maintained, suggesting that abnormal insulin secretion could be attributed to impaired anaplerosis.

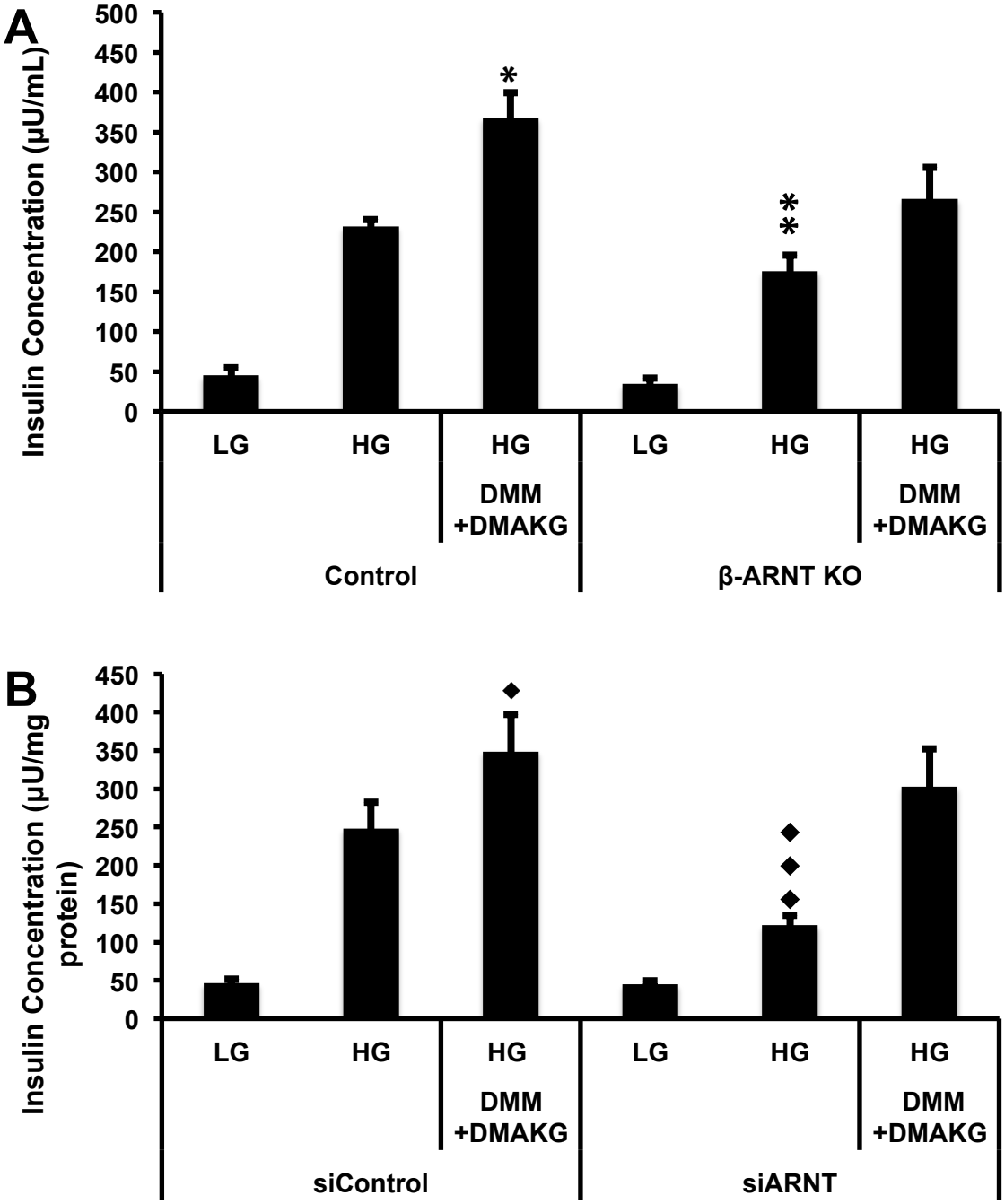

Figure 31: Effects of dimethyl malate (DMM)+dimethyl alpha-ketoglutarate (DMAKG) treatment on GSIS. A) GSIS in β-ARNT KO islets with either 2 mM (LG) or 7 mM Glucose (HG) and in the presence of 5 mM DMM+DMAKG with HG. B) GSIS in 832/13 cells treated with either siControl or siARNT with either 2 mM (LG) or 7 mM (HG) glucose and 5 mM DMM +DMAKG with HG. Results represent mean ± SEM of 3-6 independent experiments. **p<0.05 Control HG Vs Control HG +DMM+DMAKG, ** p <0.01 Control HG Vs β-ARNT KO HG; ◆p<0.05 siControl HG Vs siControl HG+DMM+DMAKG, ◆◆◆p<0.001 siControl HG Vs siARNT HG. Student's t-test.*

4.4.12 Measurement of NADPH: NADP ratio

To understand the nature of the amplifying signals that may be blunted in the absence of ARNT/HIF1β, we decided to investigate glucose induced rise in NADPH: NADP ratio in 832/13 cells treated with siARNT. It is generally believed that stimulation of insulin secretion by glucose leads to a rise in metabolic coupling factors like NADPH (67,72,91,253,254). As shown in Figure 32 A, B a two-fold rise in NADPH: NADP ratio was observed in siControl-treated 832/13 cells when glucose concentration was raised from 2mM to 16.7 mM. However, the glucose induced rise in NADPH: NADP ratio was negligible in siARNT-treated 832/13 cells.

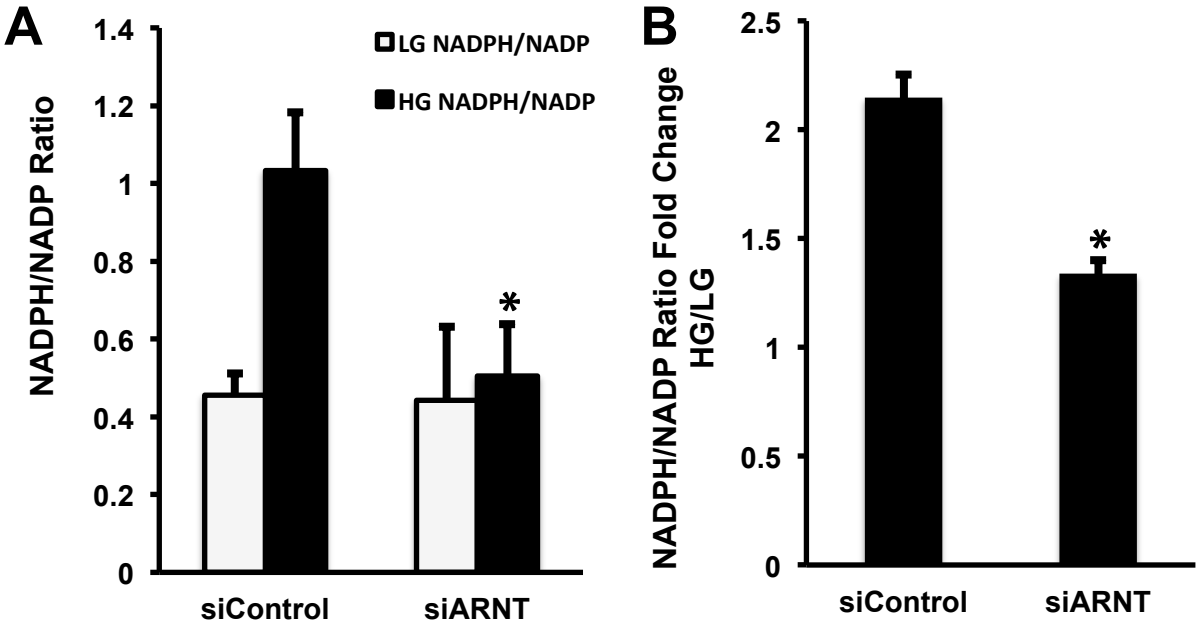

Figure 32: Effect of siARNT-treatment of NADPH: NADP ratio in 832/13 cells. A) 832/13 cells were treated with siControl or siARNT. NADPH and NADP levels were measured as described in "Experimental Procedures" in 832/13 cells exposed to either 2 mM (LG) or 16.7 mM glucose (HG). B) Data in (A) represented as HG/LG fold change in NADPH/NADP ratio in siControl and siARNT 832/13 cells. Results represent the mean ± SEM for 4 independent experiments. *$p<0.05$ *siControl Vs siARNT. Student's t-test.*

4.4.13 Intracellular Calcium Measurements in β-ARNT KO Islets

Calcium (Ca^{2+}) is yet another essential signal in the stimulus-secretion coupling that is critical to trigger insulin granule exocytosis. Ca^{2+} also plays a key role in the activation of many mitochondrial dehydrogenases that can stimulate cellular respiration (255). To investigate whether impairment in GSIS observed in β-ARNT KO islets is due to changes in intracellular Ca^{2+} levels, we measured intracellular Ca^{2+} concentrations in response to change in glucose concentration using Fura-2AM, a ratiometric Ca^{2+} indicator (Figure 33 A, B). We did not find any difference in Ca^{2+} under low glucose conditions (2 mM) between control and β-ARNT KO islets. However, upon stimulation with 20 mM glucose, intracellular Ca^{2+} levels were 26.4% less in β-ARNT KO islets compared to control islets. To see whether the reduction in intracellular Ca^{2+} could be due to changes in the activity of the voltage-gated calcium channels, direct depolarization of the beta-cell plasma membrane was induced by 30 mM KCl. We observed a small but significant reduction in intracellular Ca^{2+} levels (19.7%) in β-ARNT KO islets compared to control islets upon KCl stimulation in the presence of 20 mM glucose. Our data indicates that loss of ARNT/HIF-1β leads to a reduction in glucose induced calcium influx and it may be caused by abnormalities in the activity of the calcium channels.

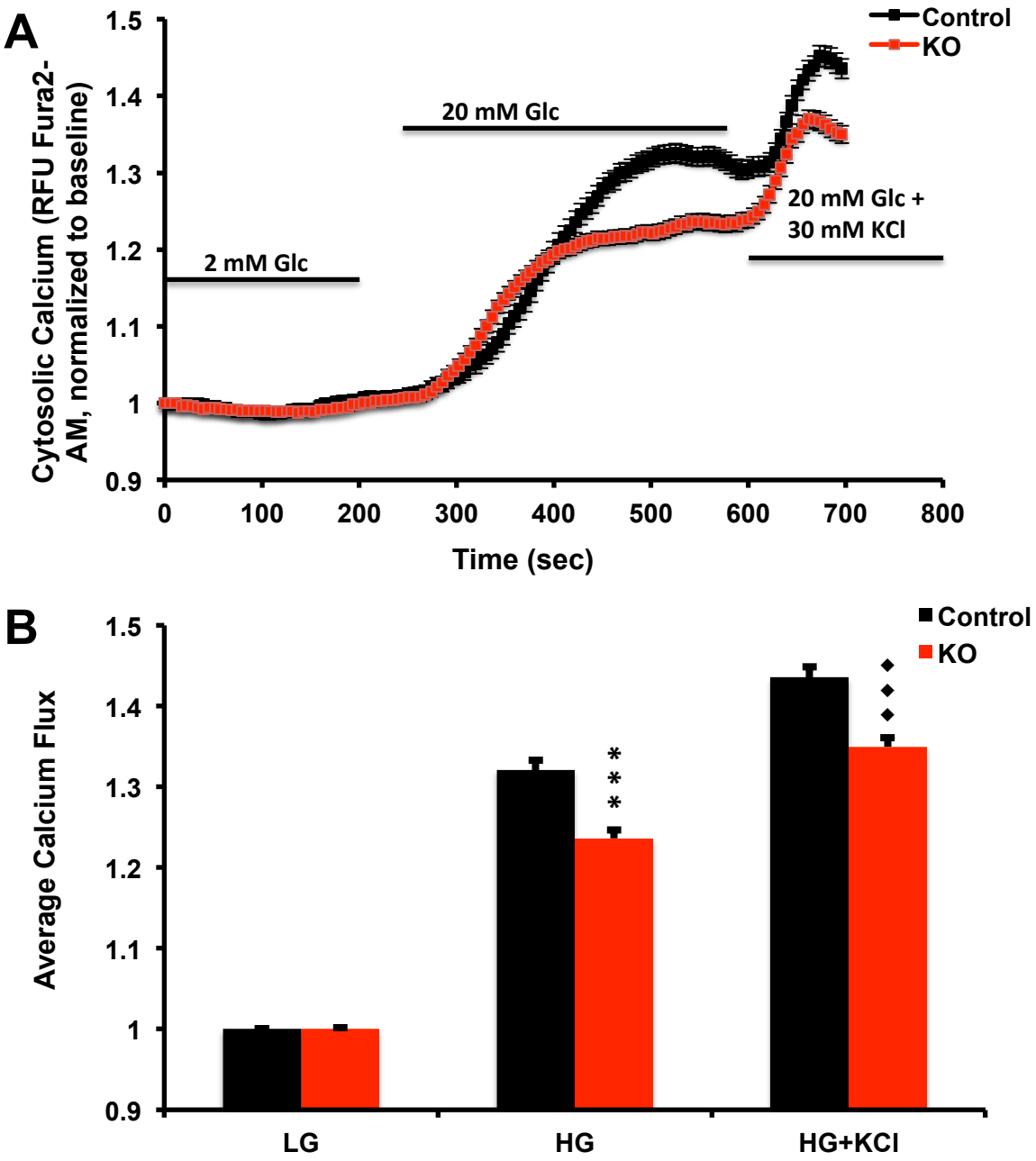

Figure 33: Changes in intracellular Ca^{2+} in β-ARNT KO islets A) Representative kinetic trace of the Fura2AM fluorescent signal from pooled β-ARNT KO islets (red trace) and control (black trace) islets; glucose (glc) B) Average calcium influx under low glucose (LG; 2 mM glucose), high glucose (HG; 20 mM glucose) and high glucose plus KCl (20 mM HG + 30 mM KCl) in control (black bar) and β-ARNT KO islets (red bar). Results represent mean ± SEM of 3 independent experiments (n=3 - 4 mice per genotype). *** *p<0.001 for HG Control vs HG β-ARNT KO islets,* ◆◆◆ *p<0.001 HG+KCl Control Vs HG+KCl β-ARNT KO islets. Student's t-test.*

4.5 DISCUSSION

In the previous chapter, we characterized the metabolic phenotype of INS-1 derived 832/13 cells where siRNA mediated knock down of ARNT/HIF-1β led to impairments in glucose-stimulated anaplerosis and insulin secretion (249). In the present study, we generated a pancreatic beta-cell specific ARNT/HIF-1β knock-out mouse using the Cre-loxP system, where the Cre expression was driven by a rat insulin promoter. Using isolated islets from β-ARNT KO mice, we further characterized the defective insulin secretion that is inherent in these islets and identified the metabolic amplifying signals that are necessary for ARNT/HIF1β-mediated GSIS. Our study indicates that ARNT/HIF-1β is critical for maintaining the normal secretory function of pancreatic beta-cells in mice. However, contrary to previous results (164) both male and female β-ARNT KO mice generated for this study have normal glucose homeostasis. Further analysis into the whole body metabolic phenotype of β-ARNT KO mice shows slightly but significantly higher preference for carbohydrate utilization leading to an increase in RER. This suggests a possible activation of a compensatory pathway in the peripheral tissues leading to normal glucose clearance and maintenance of normal glucose homeostasis in β-ARNT KO mice.

The lack of glucose intolerance in β-ARNT KO mice was an unexpected outcome of this study. Gunton et al., (164) had reported impaired glucose tolerance in both male and female β-ARNT KO mice. The difference in results could be due to the following reasons: 1) Use of specific controls – Studies have shown that presence of RIP-Cre transgene alone can lead to glucose intolerance in mice (250). Therefore, all control animals used in our study were positive for the Cre transgene. In contrast, the glucose tolerance test conducted for β-ARNT KO mice in Gunton et al's study was compared only to the floxed littermates. 2) Differences in the background strain of mice used for generating knock-out models can often lead to differences in phenotypes as observed in beta-cell specific HNF-4α knock-out mice (212,213). Further investigation is indeed needed to assess the differences observed between the β-ARNT KO mice in these two studies. Maintenance of whole body glucose homeostasis mainly depends on the uptake and storage of glucose by peripheral tissues such as skeletal

muscles, adipose tissue and liver (256). The observation that β-ARNT KO mice have higher RER compared to control mice suggests a few possibilities. Considering the fact that indirect calorimetry provides an index of overall substrate disappearance rates and not substrate oxidation rates (257), a higher RER in β-ARNT KO mice could be an indication that peripheral tissues would utilize more glucose, leading to improved glucose clearance from the blood stream. If this is the case, then it may result in changes in the glycogen reserves in the muscles of β-ARNT KO mice compared to the control mice, depending on whether glucose is utilized in the oxidative pathway or non-oxidatively stored as glycogen. In addition, our β-ARNT KO mice showed comparable or slightly improved (non-significant) insulin sensitivity in skeletal muscles leading to similar glucose tolerance as control mice. Lima et al had proposed a role for muscle HIF-1 (HIF-1α and ARNT/HIF1β) in glucose homeostasis where HIF-1 up-regulated the expression of GLUT-4 mRNA following muscle contraction (258). However, we did not study the expression of GLUT-4 in the muscles of β-ARNT KO mice. The involvement of the central nervous system (CNS) in the regulation of whole body glucose sensing in β-ARNT KO mice was also ruled out as we did not find any changes in metabolic parameters that are typically regulated by the CNS such as food intake or body weight. This suggests that hypothalamic glucose sensing may not be impaired in β-ARNT KO mice. Consistent with this, ablation of ARNT/HIF-1β in hypothalamic POMC neurons leads to overeating and obesity indicating that HIF-mediated signalling in the hypothalamus is required for maintaining glucose sensing and energy balance in mice and this was found to be mostly mediated through HIF-2α - ARNT/HIF-1β partnership (259). Further investigation is indeed necessary to assess how local inhibition of ARNT/HIF-1β can evoke compensatory responses in the peripheral tissues.

Our results with isolated islets from β-ARNT KO mice clearly indicate that ARNT/HIF-1β is necessary for normal insulin secretion. The fact that the two signals critical for insulin secretion, namely, the rise in NADPH/NADP ratio and rise in intracellular $Ca^{2+,}$ are both regulated by ARNT/HIF-1β is a new discovery. As shown earlier in this thesis, loss of ARNT/HIF-1β in 832/13 cells leads to a decrease in glucose-induced rise in TCA cycle

intermediates (249). Others have shown that reduction in mitochondrial substrate availability also leads to reduction in the generation of cytosolic NADPH (260). Therefore, loss of NADPH/NADP ratio was an expected outcome of ARNT/HIF-1β protein deletion. An increasing amount of evidence suggests that anaplerosis is very important for GSIS (72,79,240). Whether TCA cycle intermediates play a direct role or indirectly assist insulin secretion is largely unknown. However, it is important to note that some of these TCA cycle intermediates should be exported from the mitochondria to the cytosol and may have extra-mitochondrial signalling activity, where they aid in potentiating or triggering insulin secretion (65,66,68,204). NADPH has long been proposed as one such signalling molecule important for insulin secretion, as the ratio of this pyridine nucleotide increases in direct proportion with glucose and GSIS. Also, addition of NADPH has been shown to cause exocytosis of insulin granules in beta-cells (67,72,91). The fact that loss of GSIS in β-ARNT KO islets and 832/13 cells with low levels of ARNT/HIF-1β could be restored to near-normal levels by treatment with TCA cycle intermediates namely DMAKG and DMM provides further proof that ARNT/HIF-1β plays a critical role in maintaining glucose-stimulated anaplerosis and signals generated thereof in the regulation of insulin secretion from pancreatic beta-cells.

In conclusion, the data presented here clearly shows the critical role played by ARNT/HIF-1β in maintaining the secretory function of pancreatic beta-cells mainly through the regulation of anaplerosis, glucose-stimulated rise in NADPH/NADP ratio and rise in intracellular Ca^{2+} levels. *In vivo,* contrary to previous reports, loss of ARNT/HIF-1β does not subject the mice to glucose intolerance, possibly due to the activation of a compensatory mechanism in the peripheral tissues as suggested by the higher RER in the knock-out mice. However, the exact nature of this compensation is currently unknown.

Chapter 5
Summary and Conclusions

5.1 Summary of Findings

5.1.1 *In vitro* Findings from siARNT-treated 832/13 Beta-Cell Line

- The following parameters were found to be significantly reduced in siARNT-treated 832/13 cells compared to siControl-treated cells:
    1) Insulin secretion in response to glucose, IBMX and palmitate
    2) Glucose utilization or glycolytic flux
    3) Glucose-stimulated anaplerosis
    4) Glucose-stimulated rise in NADPH/NADP ratio
    5) Gene expression of PC, PDH, DIC, OGC, MEc, CPT1, FAS and GPR40
- No significant differences were observed between siARNT and siControl-treated 832/13 cells in the following parameters:
    1) Insulin secretion in response to amino acids
    2) Glucose oxidation
    3) ATP/ADP ratio

5.1.2 *In vivo* Findings from β-ARNT KO Mice

- **RER was found to be slightly, but significantly higher in β-ARNT KO mice**
- No significant differences were observed between β-ARNT KO mice and control mice in the following parameters:
    1) Body weight
    2) Fasting blood glucose
    3) Glucose tolerance
    4) Insulin sensitivity
    5) Feeding and activity

5.1.3 *In vitro* Findings from β-ARNT KO Islets

- The following parameters were found to be significantly reduced in islets from β-ARNT KO mice compared to islets from control mice:
    1) Insulin secretion in response to glucose, KCl and diazoxide
    2) Glucose-stimulated rise in intracellular calcium
    3) Gene expression of anaplerotic enzyme, PC
- No significant differences were observed between islets from β-ARNT KO and control mice in the following parameters:
    1) Insulin secretion in response to DMM, DMAKG in the presence of glucose
    2) Glucose oxidation
    3) Mitochondrial membrane potential

5.2 Conclusions

The alarming rise in the incidence of T2D across the globe calls for better understanding of the disease and development of better therapeutic options to manage or delay the onset of the disease. Pancreatic beta-cell dysfunction leading to decreased insulin secretion is one of the hallmark features of T2D. In addition, people with T2D also have lowered sensitivity to insulin in the peripheral tissues such as the skeletal muscles, the liver and the adipose tissue, leading to whole body insulin resistance. Indeed, improving insulin sensitivity and increasing insulin secretion by the pancreatic beta-cells require proper understanding of the mechanisms that govern these processes in our body. Therefore, before we begin to develop new therapeutics for the treatment of T2D, it is imperative that we understand the biology of the pancreatic beta-cells and the basic mechanisms that regulate glucose-stimulated insulin secretion (GSIS).

The mechanism of GSIS from pancreatic beta-cells has been well studied for the past several decades. Unlike hormones and neurotransmitters, which binds their beta-cell receptors and

stimulates insulin secretion through the classical PKA or PKC signal transduction pathway, the mechanism that governs GSIS from pancreatic beta-cells is mainly dependent on its ability to act as a fuel sensor, metabolize these fuels and adapt to the variation in plasma concentration of glucose and other nutrients (56,200,261). Accordingly, the best-characterized pathway of GSIS, also known as the K_{ATP} dependent pathway, produces the triggering signals, namely, the rise in ATP/ADP ratio and increase in intracellular Ca^{2+}, for the exocytosis of insulin granules. A second pathway that has gained wide popularity is the K_{ATP} independent pathway, which is largely based on the ability of glucose to amplify insulin secretion even in the absence of a functional K_{ATP} channel (64,262). Here, it is generally believed that glucose does not cause any further increase in intracellular Ca^{2+}, but increases its efficacy on the exocytotic machinery. It is also believed that mitochondrial metabolism of glucose generates signals other than ATP, which play a critical role in further amplification of insulin granule exocytosis. Although the existence of this pathway is widely accepted, the underlying mechanisms that govern this pathway are still unclear.

Dissection of the transcriptional network and genetic regulators of pancreatic beta-cells has led to the identification of a number of genes that are critical for proper maintenance of beta-cell function and insulin secretion. One potentially important player in the pathogenesis of beta-cell dysfunction leading to T2D is ARNT/HIF1β. Since its discovery in 2005 as the most down-regulated gene in human type 2 diabetic islets and as a positive regulator of insulin secretion, a number of research groups have evaluated its role, not just in the beta-cells, but also in other metabolically active tissues in our body. The main objective of our study was to investigate the metabolic phenotype of beta-cells deficient in ARNT/HIF-1β and understand how the transcription factor regulates GSIS from the beta-cells and assists in the maintenance of glucose homeostasis in mice.

5.2.1 Conclusions from siARNT-treated 832/13 cells and β-ARNT KO Islets

Consistent with its role as a positive regulator of glucose metabolism, we found that loss of ARNT/HIF-1β in 832/13 cells led to a significant reduction in GSIS and glucose-stimulated rise in glycolysis. However, we found that this reduction in the rate of glycolysis neither

affected the rate of glucose oxidation nor the amount of ATP synthesized by the beta-cell mitochondria. These data clearly demonstrated that the mechanism of regulation of GSIS by ARNT/HIF-1β was independent of rise in ATP/ADP ratio, which is one of the most important triggering signals required for GSIS from beta-cells. This also suggested that regulation of GSIS by ARNT/HIF-1β was mediated through mechanisms or pathways that are independent of the K_{ATP} channel function. As mentioned previously, studies conducted by multiple groups had suggested the importance of mitochondrial pathways of glucose metabolism and the signals generated by these pathways in the regulation of GSIS. To gain a better understanding of the metabolic pathways that are regulated by ARNT/HIF1β, a metabolite profiling of 832/13 cells with low ARNT/HIF-1β levels was performed using GC-MS. The results yielded from this study were very dramatic as we discovered a significant decrease in most glycolysis and TCA cycle intermediates in the absence of ARNT/HIF1β. Not only did the loss of ARNT/HIF-1β affects glucose metabolism pathway, but it also negatively impacted the fatty acids and the amino acids pathway. On further investigation, it was revealed that ARNT/HIF-1β regulates a number of genes that are critical for the maintenance of the anaplerotic pathway and pyruvate cycling such as PC, PDH, DIC, OGC and MEc, which were all shown to be crucial for the maintenance of GSIS from the beta-cells. Especially, the down-regulation of PC, the critical regulator of anaplerosis, was significant in both siARNT-treated 832/13 cells and β-ARNT KO islets. Loss of PC expression has a severe impact on GSIS as demonstrated by siRNA and pharmacological inhibition of PC in 832/13 cells as well as in rat islets (70,72,246,263,264). PC mediated anaplerosis not only helps to export the TCA intermediates from the mitochondria to the cytoplasm where they serve as amplifying signals for insulin secretion, but studies have shown that loss of PC decreased both first and second phase insulin secretion and ATP/ADP ratio in rat islets suggesting that it helps in the generation of sufficient ATP to regulate both phases of GSIS. Consistent with the observation that ARNT/HIF-1β regulates the PC-mediated anaplerotic pathway, it was not surprising to see that loss of ARNT/HIF-1β tends to have a negative impact on NADPH/NADP ratio. NADPH is an expected by-product of all three pyruvate cycling pathways and is considered to be one of the most important signalling

molecules generated by the mitochondrial metabolism of glucose for the maintenance of the amplifying pathway of GSIS. Taken together, our results from 8321/13 cells clearly suggest a critical role for ARNT/HIF-1β in maintaining GSIS from pancreatic beta-cells.

The biochemical characterization of β-ARNT KO islets also yielded similar results as observed in 8321/3 cells and clearly suggested the involvement of ARNT/HIF-1β in the regulation of GSIS through amplifying or the K_{ATP} independent pathway of insulin release. Islets obtained from both male and female β-ARNT KO mice showed severe and significant impairments in GSIS. Monitoring β-ARNT KO islets for changes in mitochondrial membrane potential in response to glucose, which also serves as a surrogate marker for ATP production, yielded results that implied the lack of involvement of ARNT/HIF-1β in the regulation of the ATP-dependent triggering pathway of GSIS. This was further confirmed by the results obtained from the oxygen consumption studies using Seahorse flux analyzer in β-ARNT KO islets, where the rate of oxygen consumption in β-ARNT KO islets in response to glucose, yet another indicator of cellular ATP production, was found to be similar in control and knock-out islets. Insulin secretion studies using KCl and diazoxide were used to investigate the functionality of the amplifying pathway or the K_{ATP} independent pathway in β-ARNT KO islets and the results indicated a defect in this pathway. Consistent with this, addition of anaplerotic and pyruvate cycling intermediates DMAKG and DMM restored insulin secretion in β-ARNT KO islets, suggesting the involvement of anaplerosis in the regulation of GSIS mediated by ARNT/HIF1β. Both alpha ketoglutarate and malate are part of the pyruvate cycling pathways, which aid in the influx of pyruvate to the mitochondria to enter the TCA cycle and promote the efflux of TCA intermediates such as citrate and malate for the generation of amplifying signals (70,245,265). Besides the fact that DMAKG and DMM are the most widely used methyl esters of alpha-ketoglutarate and malate, one of the compelling reasons to use them in our study was the fact that both these TCA intermediates were found to be critically low in 832/13 cells in the absence of ARNT/HIF1β. Use of DMM has previously been shown to increase GSIS through increasing pyruvate cycling and anaplerosis (70). Alpha-ketoglutarate serves several functions in pancreatic beta-cells. It helps in the proper maintenance of the malate-aspartate shuttle and in the transportation of

NAD^+ from the mitochondria to the cytosol for its re-use in glycolysis (71,266). Research also suggests that there is no fuel insulin-secretagogue that is incapable of producing alpha-ketoglutarate and in addition to its role in the maintenance of TCA cycle and as a precursor for anaplerosis, it may also have a signalling role in the beta-cells. Specifically, alpha-ketoglutarate is a substrate for several alpha-ketoglutarate hydroxylases or dioxygenases, which are involved in the post-translational hydroxylation of proteins (225,267). Taken together, the recovery of insulin secretion by DMAKG and DMM suggest that loss of GSIS in β-ARNT KO islets is partially mediated through alterations in the metabolic fate of pyruvate and the associated signalling molecules generated from potential pyruvate cycling pathways.

One of the main findings from the studies using β-ARNT KO islets is the significant decrease in the influx of intracellular Ca^{2+} in response to high glucose as well as high glucose and KCl. The triggering Ca^{2+} signalling is an essential part of GSIS, which ensures the exocytosis of insulin granules (242). The importance of Ca^{2+} in GSIS was further strengthened by the observation that any experimental condition that interferes with the rise in intracellular Ca^{2+} impairs GSIS, whereas physiological or pharmacological agents, that increase beta-cell intracellular Ca^{2+}, regardless of the mechanism, induce insulin secretion (268). In fact, many of the T2D drugs used to enhance insulin secretion thrive on this mechanism (269). Drugs such as the sulfonylureas, stimulate insulin secretion by binding to the beta-cell K_{ATP} channels, depolarizing the plasma membrane, inducing a rise in intracellular Ca^{2+}, which then triggers the exocytosis of insulin granules. Although the supremacy of the K_{ATP} channel dependent pathway in the stimulation of intracellular Ca^{2+} influx is very evident, it should be noted that promotion of Ca^{2+} influx is not always a K_{ATP} channel dependent event (58). Insulin secretagogues such as KCl or amino acids such as arginine or alanine can cause beta-cell membrane depolarization and promote Ca^{2+} influx without affecting the K_{ATP} channels. Also, studies from mice lacking K_{ATP} channels SUR1 or Kir6.2 in beta-cells indicate that glucose can still elicit insulin secretion in a K_{ATP} independent manner by promoting membrane depolarization and influx of Ca^{2+} (63). The Ca^{2+} data obtained from β-ARNT KO islets in the presence of high glucose and high KCl indicates possible defects in the voltage

dependent Ca^{2+} channels and points to the possibility that defects may be independent of the K_{ATP} channels. The essential role played by L-type Ca^{2+} channels in the influx of extracellular Ca^{2+} is undisputable (270). However, a concerted activation of a variety of channels contributes to Ca^{2+} influx during the triggering and the amplifying phase of insulin release. Particularly, it has been shown that genetic or pharmacological inhibition of the R-type channels ($Ca_V2.3$) lead to a significant reduction in the second phase or the amplifying phase of GSIS both *in vitro* and *in vivo* in mice (271). Reduced Ca^{2+} response to KCl in β-ARNT KO islets could also be therefore attributed to a defect in the channel activity of the R-type channels, however, this was not further investigated in our study.

Overall, our study clearly shows that ARNT/HIF-1β regulates GSIS predominantly through the K_{ATP} independent pathway of insulin release and that it is required to maintain glucose-stimulated anaplerosis and generation of anaplerotic signal, NADPH. In addition, ARNT/HIF-1β is required to maintain the influx of intracellular Ca^{2+} in response to glucose, most likely independent of the activity of the K_{ATP} channels.

5.2.2 Conclusions from β-ARNT KO Mice

The unexpected outcome of this study was the lack of a diabetic phenotype in both male and female β-ARNT KO mice. β-ARNT KO mice were born at expected Mendelian ratios and did not differ in size or weight from their control littermates. Metabolic parameters that are indicative of abnormal glucose homeostasis such as fasting blood glucose measurements and the intraperitoneal glucose tolerance test were normal and comparable to control mice, indicating that secretory abnormalities observed in islets from β-ARNT KO mice did not translate to glucose intolerance in our model of mice. This was quite an interesting outcome since Gunton et al had previously shown that both male and female β-ARNT KO mice were glucose intolerant (164). Based on the fact that ARNT/HIF-1β is a metabolic regulator and to determine whether beta-cell specific deletion of ARNT/HIF-1β has any impact on the whole body metabolic phenotype, indirect calorimetry was used in β-ARNT KO mice to measure complex metabolic parameters such as food intake, activity, energy expenditure, volume of O_2 (VO_2) consumed and CO_2 (VCO_2) expelled and respiratory exchange ratio (RER) under

ad libitum conditions. Except for RER which we found to be slightly but significantly increased in β-ARNT KO mice, we did not find any significant changes in other metabolic parameters in β-ARNT KO mice. An increase in RER (VCO_2/VO_2) and a value that is closer to 1 in β-ARNT KO mice is indicative of increased carbohydrate utilization compared to control mice. Typically RER values are also indicative of metabolic flexibility, which is the body's capacity to match fuel oxidation to fuel availability (272). A higher RER in the post-prandial or fed state, conditions under which β-ARNT KO mice were analyzed, is often associated with increased sensitivity to insulin and lower RER is associated with obesity, insulin resistance and T2D. T2D patients are often found to metabolically inflexible, meaning, they are unable to efficiently switch to carbohydrate oxidation in the post-prandial state and often have to depend on oxidation of lipids for energy expenditure, reducing the RER values (273,274). Based on this, an increased RER in β-ARNT KO mice also suggests two things 1) increased sensitivity of muscle tissues to insulin, leading to better glucose disposal 2) skeletal muscles in β-ARNT KO mice are metabolically more flexible than control mice, protecting them from hyperglycemia. Although our insulin tolerance test did not show a statistically significant increase in overall insulin sensitivity in both male and female β-ARNT KO mice, these mice generally responded better to exogenous insulin compared to their control littermates and statistically significant results were obtained at 60 minutes in male β-ARNT KO mice, indicating better insulin stimulated glucose uptake. Results from both RER and insulin tolerance tests were indicative of improved functioning of the skeletal muscles, as systemic substrate use is largely dependent on the muscles compared to any other metabolically active peripheral organs in our body. Thus, based on the data generated from this study, disruption of ARNT/HIF-1β in pancreatic beta-cells does not lead to glucose intolerance in mice. Although islets from β-ARNT KO mice secrete less insulin compared to their controls, these mice seemed to be protected from hyperglycemia through increased utilization of carbohydrates through the skeletal muscles possibly leading to improvements in overall glucose clearance from the body.

5.3 Does Loss of ARNT/HIF-1β in Pancreatic Beta-cells Play a Role in the Progression of T2D?

T2D generally results from the inability of pancreatic beta-cells to secrete enough insulin over time to compensate for the insulin resistance that is evident in our body. Maintenance of normal beta-cell function requires the expression of key transcription factors, which assist in the optimal expression of genes that are critical for mature beta-cell function such as maintenance of beta-cell mass and insulin secretion. This is supported by several studies where the functional significance of these transcription factors has been critically evaluated through loss of function or gain of function analysis, both *in vitro* and *in vivo* (207,275,276). Our *in vitro* data from 832/13 cells and β-ARNT KO islets clearly indicates a critical role for ARNT/HIF-1β in maintaining normal beta-cell function and insulin secretion. As observed in Gunton's study (164), where ARNT/HIF-1β was found to regulate key genes involved in glucose metabolism, our study also recognizes the importance of ARNT/HIF-1β in maintaining the expression of several key genes involved in both arms of glucose metabolism, namely, glycolysis and the TCA cycle, thereby regulating glucose-stimulated anaplerosis and GSIS from pancreatic beta-cells. However, the *in vivo* data obtained from β-ARNT KO mice in our study was quite a contrast to the data obtained from Gunton's study where both male and female β-ARNT KO mice were found to be glucose intolerant. It was indeed intriguing to see that the *in vitro* impairment in GSIS in the absence of ARNT/HIF-1β did not translate to an *in vivo* diabetic phenotype resulting in glucose intolerance and hyperglycemia as reported by Gunton's study. Not only did our model of β-ARNT KO mice have normal glucose homeostasis, but we discovered that maintenance of normal glucose homeostasis was achieved through a small but significant up-regulation of carbohydrate fuel oxidation in the skeletal muscles. Therefore, based on our results, loss of ARNT/HIF-1β specifically in pancreatic beta-cells does not lead to abnormal glucose homeostasis, resulting in type 2 diabetes in mice. However, our study has presented compelling evidence showing the requirement of ARNT/HIF-1β in maintaining normal glucose-stimulated insulin secretion and normal beta-cell function in mice. Over time, lack of ARNT/HIF-1β may severely impact beta-cell function creating a perfect storm for the genesis of type 2 diabetes.

Collectively, the results obtained from site-specific deletions of ARNT/HIF-1β in metabolically active tissues such as the pancreas, the liver and the adipose tissue, indicates an important role for this transcription factor in maintaining metabolic homeostasis (164,170,173,183). Given the known alteration of ARNT/HIF-1β and its binding partner HIF-1α in diabetic pancreatic beta-cells and diabetic liver of humans, it is quite reasonable to speculate that the HIF-signalling pathway plays a central role in the pathogenesis of type 2 diabetes in humans as seen in mice. However, more studies are definitely needed for an in-depth understanding of the complexities involved in HIF-1 signalling and to explain the phenotypic and metabolic disparities that exist in mice models generated by different research groups where HIF-1 signalling is impaired as observed in adipocytes and pancreatic beta-cells specific HIF-1α and ARNT/HIF-1β knock-out mice.

5.4 Future Directions

5.4.1 Further Studies on β-ARNT KO Mice

1. ***In vivo* Insulin Measurements:** Plasma insulin measurement is an important indicator of whole body glucose homeostasis and progression of type 2 diabetes. Although islets from β-ARNT KO mice secreted significantly less insulin compared to their control littermates, these mice maintained normal glucose homeostasis. Therefore, it will be interesting to measure the plasma insulin levels, as it will add an additional layer to defining the mechanistic role of ARNT/HIF-1β in maintaining normal glucose homeostasis.
2. **Examining Skeletal Muscle Glucose Uptake and Storage in β-ARNT KO Mice:** Increased RER in β-ARNT KO mice is suggestive of higher glucose uptake and utilization by the muscle tissue. However, we did not investigate whether this is true for β-ARNT KO mice and if it is, then what would be the mechanism behind improved glucose uptake by the muscles in β-ARNT KO mice. Skeletal muscles dispose off glucose through the oxidative pathway or the non-oxidative pathway, where it is stored as glycogen. A very small amount of glucose is also stored as fat via

de novo lipogenesis in the skeletal muscles. Studies have shown that, in normal people, 80% of the ingested glucose is stored in the muscle as glycogen (36,277). Studies using hyperglycemic - hyperinsulinemic clamps in type 2 diabetic patients have shown that rate of glycogen synthesis in type 2 diabetic patients is 50% less than that observed in normal healthy individuals (278). Insulin stimulated glucose uptake by skeletal muscles is largely dependent on the uptake of glucose by the insulin-responsive glucose transporter, GLUT4. Once inside the cell, glucose is phosphorylated by hexokinase, converting it to hexose 6-phosphate. Hexose 6-phosphate is then utilized to enter the oxidative pathway or the non-oxidative pathway where it is converted to muscle glycogen by glycogen synthase. ^{13}C magnetic resonance studies have shown that transport of glucose by GLUT 4 is the rate-limiting step in glucose uptake process by the skeletal muscles (279). Studies have also shown that insulin-stimulated glucose uptake is dependent on the translocation of GLUT 4 from the endosomal compartment to the plasma membrane facilitating the uptake of glucose into the muscle cell for its phosphorylation by the hexokinase (280). Based on the increased RER values obtained from β-ARNT KO mice, it may be reasonable to expect muscle glucose uptake to be slightly but significantly better in β-ARNT KO mice compared to their controls. It will also be interesting to determine the level of expression of GLUT 4 in the muscles of β-ARNT KO mice as increased responsiveness of the muscle tissue to glucose may also be due to increased expression of GLUT 4. These few pieces of information will provide a more complete story of the observed normoglycemia in β-ARNT KO mice.

3. **β-ARNT KO Mice and High Fat Diet:** A chronic high fat diet (HFD) will be an interesting way to investigate whether environmentally induced metabolic stress can impact the metabolic phenotype of β-ARNT KO mice. With dysfunctional beta-cells, it will be interesting to see whether β-ARNT KO mice are protected from the adverse effects of HFD as observed in adipocyte-specific ARNT/HIF-1β knock-out mice (183) or they will be unable to cope with the increased energy demand. The adipocyte specific deletion of ARNT/HIF-1β and HIF-1α in mice leads to protection from the

consequences of HFD, which was attributed to an increase in insulin signalling in adipose tissue, the liver and the muscles. The adipocyte-specific HIF-1α mice also exhibited central effects with an increase in core temperature and energy expenditure, suggesting a cross-talk between the adipose tissue and the brain. On a normal chow diet, we did not observe a similar involvement of the central nervous system (CNS) in β-ARNT KO mice as there were no changes in metabolic parameters that are regulated by the CNS such as food intake and body weight. However, it will be interesting to see whether the same protection mechanisms will be activated in β-ARNT KO mice under HFD.

5.5 Final Remarks

Based on our biochemical and molecular characterization of 832/13 cells treated with siRNA against ARNT/HIF-1β and islets from β-ARNT KO mice, we believe that ARNT/HIF-1β plays a central role in the maintenance of glucose-stimulated insulin secretion from the pancreatic beta-cells. The mechanism of insulin release mediated through ARNT/HIF-1β is independent of the K_{ATP} channels and increase in cellular ATP/ADP ratio, critical for the triggering of insulin granule exocytosis. However, ARNT/HIF-1β seems to play an important role in maintaining the K_{ATP}-independent or the amplifying pathway of insulin release, regulating glucose-stimulated anaplerosis and the key enzymes that are important to maintain normal substrate flow through the TCA cycle. Important stimulus-secretion coupling factors such as NADPH and Ca^{2+} seems to be impacted by the loss of ARNT/HIF1β, suggesting a need for the optimal expression of the transcription factor for the maintenance of metabolic signals critical for insulin secretion. *In vivo*, loss of ARNT/HIF-1β does not seem to affect glucose homeostasis in mice and β-ARNT KO mice seems to be protected from hyperglycemia through a small but significant increase in carbohydrate utilization, represented by the increased respiratory exchange ratios, the mechanism of which is currently unknown (Figure 34). Overall, our data demonstrates a critical need for the optimal expression of ARNT/HIF-1β in maintaining normal beta-cell function and we believe that our study provides significant information necessary for assessing the pharmacological potential of gene targets for the treatment of type 2 diabetes.

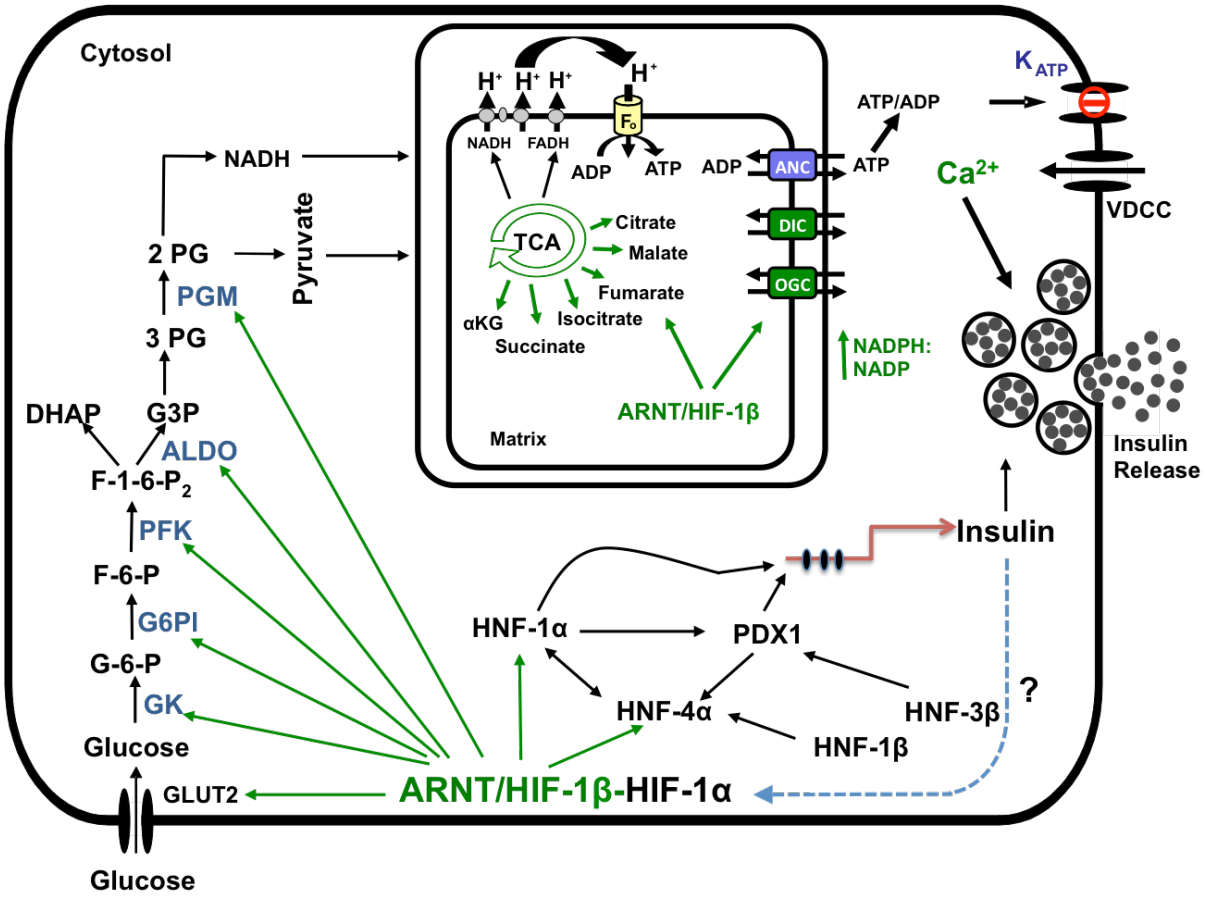

Figure 34: Schematics of the transcriptional network regulated by ARNT/HIF-1β and its involvement in GSIS. ARNT/HIF-1β regulates the expression of key enzymes in glycolysis and TCA cycle and regulates the expression of mitochondrial metabolite carrier proteins (denoted by green arrows and green boxes), which plays an important role in the generation of cytosolic second messengers such as NADPH. ARNT/HIF-1β has also shown to influence the expression of MODY genes, HNF-1α and HNF-4α making it a master regulator of beta-cell transcriptional network. Metabolic processes such as anaplerosis, generation of NADPH and influx of Ca^{2+}, critical for maintaining GSIS, are shown to be regulated by ARNT/HIF-1β. Insulin has been shown to regulate several metabolic genes through ARNT/HIF-1β-HIF-1α heterodimers, however, this has yet to be demonstrated in pancreatic beta-cells.

Appendix A

Biography

Educational Qualification:

PhD (Biology)	**School of Pharmacy, University of Waterloo**	**2009-2014**
MSc (Biology)	**Department of Biology, University of Ottawa**	**2003-2005**

Publications:

1. Casimir M, Dai XQ, **Pillai R**, Hajmrle C, Joseph JW, Oudit JY, MacDonald PE **(2014).** Gelsolin controls insulin secretion through actin dependent and independent mechanisms **– Revisions in Diabetologia**
2. **Pillai R,** Huypens P, Huang M, Schaefer S, Sheinin T, Wettig SD, Joseph JW **(2011).** Aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor-1β plays a critical role in maintaining glucose-stimulated anaplerosis and insulin release from pancreatic beta-cells. **Journal of Biological Chemistry, 286(2): 1014-24.**
3. Huypens P, **Pillai R,** Sheinin T, Schaefer S, Huang M, Odegaard ML, Ronnebaum SM, Wettig SD, Joseph JW **(2011)** The dicarboxylate carrier plays a role in mitochondrial malate transport and in the regulation of glucose-stimulated insulin secretion from rat pancreatic beta cells**. Diabetologia, 54(10): 135-45.**
4. **Pillai R**, Coverdale LE, Dubey G, Martin CC (2004), Histone deacetylase 1 (HDAC-1) required for the normal formation of craniofacial cartilage and pectoral fins of zebrafish. **Developmental Dynamics 231(3): 647-654**

Manuscripts in Progress:

1. **Pillai R**, Prentics K, Huang M, Bombadier E, Cousteils K, Tupling R, Wheeler M, Joseph JW, **(2014).** ARNT/HIF-1β is indispensible for maintaining beta-cell secretory function, but not for maintaining normal glucose homeostasis in mice **– Manuscript ready for submission to Journal of Biological Chemistry.**
2. Huang M, **Pillai R,** Wong J, Joseph JW **(2014).** Hydroxylation an important mechanism in the regulation of glucose metabolism – **Manuscript ready for submission.**
3. Paglialunga S, Simnett G, Jain SS, Herbst EA, Snook L, Arkell AM, **Pillai R,** Dyck DJ, Simpson JA, Bonen A, Joseph JW, Holloway GP **(2014).** The Rab-GTPase activating protein, TBC1D1, is critical for maintaining normal glucose homeostasis and β-cell mass - **Manuscript ready for submission to Cell Metabolism.**

Book Chapter:

1. **Renjitha Pillai** and Jamie W Joseph **(2012).** Distinct role for ARNT/HIF-1β in pancreatic beta-cell function, Insulin Secretion and Type 2 Diabetes. In tech; Biochemistry; ISBN 978-953-51-0076-8 – **INVITED REVIEW**

Scholarships and Awards:

1. Pharmacy Graduate Student Award (Mentoring), PhD (2013) - $ 500
2. NSERC Doctoral Scholarship, PhD (2011 -2013) - $ 42,000
3. Canadian Diabetes Association Doctoral Award, PhD (2011-2014) - $ 63,000 – *(Declined and accepted the NSERC Doctoral award)*
4. Canadian Diabetes Association Incentive Funding, PhD (2011-2014) - $ 15,000
5. University of Waterloo President's Scholarship, PhD (2011-2013) - $ 15,000
6. Ontario Graduate Scholarship, MSc (2004-2005) - $ 15,000
7. University of Ottawa Excellence Scholarship, MSc (2004-2005) - $ 6000
8. Faculty of Graduate and Postdoctoral Studies Scholarship, MSc, University of Ottawa (2003) – $ 2000
9. University of Ottawa Graduate Admission Scholarship, MSc (2003) - $2000

Conferences and Presentations:

1. **Renjitha Pillai.** Mechanistic Role of ARNT/HIF-1β in glucose-stimulated insulin secretion. – Graduate Students Research Conference, University of Waterloo – April 25, 2012 – **ORAL PRESENTATION – UNIVERSITY CONFERENCE**.
2. **Renjitha Pillai –** Type 2 Diabetes: Metabolic Regulation of Insulin Secretion –Department of Zoology, University of Calicut, Kerala, India – April 11, 2012 - **Invited Talk**
3. **Renjitha Pillai*,** Mei Huang, Peter Huypens, Tanya Sheinin and Jamie Joseph. ARNT/HIF-1β regulated glucose-stimulated insulin secretion in a K_{ATP} dependent and independent manner. WISE INITIATIVE, Celebrating 100th year of Women in Science. McMaster University, Hamilton – March 12- 2011 – **POSTER PRESENTATION – PROVINCIAL CONFERENCE.**
4. **Renjitha Pillai*** and Jamie Joseph. Replenishment of TCA intermediates in pancreatic β-cells deficient in ARNT)/HIF1β expression rescues the defective insulin secretion. American Diabetes Association 71st Scientific Sessions, San Diego Convention Center, San Diego, California, June 24-28, 2011 – **POSTER PRESENTATION – INTERNATIONAL CONFERENCE – SELECTED for Guided audio poster tour**
5. **Renjitha Pillai,** Mei Huang, Peter Huypens, Tanya Sheinin and Jamie Joseph*. ARNT/HIF-1β regulated glucose-stimulated insulin secretion in a K_{ATP} dependent and independent manner.

Keystone Symposium on Islet Biology: Whistler Conference Centre, Whistler, British Columbia - April 12 - 17, 2010 – **POSTER PRESENTATION – INTERNATIONAL CONFERENCE.**

6. **Renjitha Pillai,** Louise Coverdale, Gayatri Dubey and C.Cristofre Martin (2004). Histone deacetylase 1 (HDAC-1) required for the normal formation of craniofacial cartilage and pectoral fins of zebrafish. 63rd Annual meeting of the Society of Developmental Biology, University of Calgary, Alberta, July 24th-28th, 2004 – **POSTER PRESENTATION – INTERNATIONAL CONFERENCE**
7. **Renjitha Pillai**, Louise Coverdale, Gayatri Dubey and C.Cristofre Martin (2004). Histone deacetylase (HDAC-1) required for the formation of craniofacial cartilage and pectoral fins in zebrafish. 13th Annual Fish Physiology and Biochemistry Workshop, Keene, Ontario. February 6-8, 2004 – **POSTER PRESENTATION – REGIONAL CONFERENCE**

Bibliography

1. Eknoyan, G., and Nagy, J. (2005) A history of diabetes mellitus or how a disease of the kidneys evolved into a kidney disease. *Advances in chronic kidney disease* **12**, 223-229
2. Polonsky, K. S. (2012) The past 200 years in diabetes. *The New England journal of medicine* **367**, 1332-1340
3. Shier, D., Butler, J., and Lewis, R. (2013) *Hole's human anatomy & physiology*, 13th ed., McGraw-Hill, New York
4. Cabrera, O., Berman, D. M., Kenyon, N. S., Ricordi, C., Berggren, P. O., and Caicedo, A. (2006) The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 2334-2339
5. Levetan, C. S., and Pierce, S. M. (2013) Distinctions between the islets of mice and men: implications for new therapies for type 1 and 2 diabetes. *Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists* **19**, 301-312
6. Bosco, D., Armanet, M., Morel, P., Niclauss, N., Sgroi, A., Muller, Y. D., Giovannoni, L., Parnaud, G., and Berney, T. (2010) Unique arrangement of alpha- and beta-cells in human islets of Langerhans. *Diabetes* **59**, 1202-1210
7. Rodriguez-Diaz, R., Abdulreda, M. H., Formoso, A. L., Gans, I., Ricordi, C., Berggren, P. O., and Caicedo, A. (2011) Innervation patterns of autonomic axons in the human endocrine pancreas. *Cell metabolism* **14**, 45-54
8. Langerhans, P., and Morrison, H. (1937) *Contributions to the microscopic anatomy of the pancreas*, The Johns Hopkins press, Baltimore,
9. Banting, F. G. (1922) *Preliminary studies on the physiological effects of insulin*, Printed for the Royal Society of Canada, Ottawa,
10. Banting, F. G., and Best, C. H. (1922) *The internal secretion of the pancreas*, The University Library: pub. by the librarian, Toronto
11. Banting, F. G., and Best, C. H. (1922) *Pancreatic extracts*, The University Library: pub. by the librarian, Toronto
12. Banting, F. G., Best, C. H., Collip, J. B., Campbell, W. R., and Fletcher, A. A. (1922) *Pancreatic extracts in the treatment of diabetes mellitus*, The University library: pub. by the librarian, Toronto
13. Sanger, F., and Tuppy, H. (1951) The amino-acid sequence in the phenylalanyl chain of insulin. 2. The investigation of peptides from enzymic hydrolysates. *The Biochemical journal* **49**, 481-490
14. Sanger, F., and Tuppy, H. (1951) The amino-acid sequence in the phenylalanyl chain of insulin. I. The identification of lower peptides from partial hydrolysates. *The Biochemical journal* **49**, 463-481
15. Wilcox, G. (2005) Insulin and insulin resistance. *The Clinical biochemist. Reviews / Australian Association of Clinical Biochemists* **26**, 19-39
16. American Diabetes, A. (2011) Diagnosis and classification of diabetes mellitus. *Diabetes care* **34 Suppl 1**, S62-69
17. Lee, A. J., Hiscock, R. J., Wein, P., Walker, S. P., and Permezel, M. (2007) Gestational diabetes mellitus: clinical predictors and long-term risk of developing type 2 diabetes: a retrospective cohort study using survival analysis. *Diabetes care* **30**, 878-883

18. Cnop, M., Welsh, N., Jonas, J. C., Jorns, A., Lenzen, S., and Eizirik, D. L. (2005) Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes* **54 Suppl 2**, S97-107
19. Kaufman, D. L., Erlander, M. G., Clare-Salzler, M., Atkinson, M. A., Maclaren, N. K., and Tobin, A. J. (1992) Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *The Journal of clinical investigation* **89**, 283-292
20. Kaufman, P. A., Weinberg, J. B., and Greene, W. C. (1992) Nuclear expression of the 50- and 65-kD Rel-related subunits of nuclear factor-kappa B is differentially regulated in human monocytic cells. *The Journal of clinical investigation* **90**, 121-129
21. Ludvigsson, J., Faresjo, M., Hjorth, M., Axelsson, S., Cheramy, M., Pihl, M., Vaarala, O., Forsander, G., Ivarsson, S., Johansson, C., Lindh, A., Nilsson, N. O., Aman, J., Ortqvist, E., Zerhouni, P., and Casas, R. (2008) GAD treatment and insulin secretion in recent-onset type 1 diabetes. *The New England journal of medicine* **359**, 1909-1920
22. Atkinson, M. A. (2012) The pathogenesis and natural history of type 1 diabetes. *Cold Spring Harbor perspectives in medicine* **2**
23. Chen, W., Xie, A., and Chan, L. (2013) Mechanistic basis of immunotherapies for type 1 diabetes mellitus. *Translational research : the journal of laboratory and clinical medicine* **161**, 217-229
24. Gan, M. J., Albanese-O'Neill, A., and Haller, M. J. (2012) Type 1 diabetes: current concepts in epidemiology, pathophysiology, clinical care, and research. *Current problems in pediatric and adolescent health care* **42**, 269-291
25. Witkowski, P., and Herold, K. C. (2007) Islet transplantation for type 1 diabetes--where should we go? *Nature clinical practice. Endocrinology & metabolism* **3**, 2-3
26. Muoio, D. M., and Newgard, C. B. (2008) Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nature reviews. Molecular cell biology* **9**, 193-205
27. Rhodes, C. J. (2005) Type 2 diabetes-a matter of beta-cell life and death? *Science* **307**, 380-384
28. Gerich, J. E. (2002) Is reduced first-phase insulin release the earliest detectable abnormality in individuals destined to develop type 2 diabetes? *Diabetes* **51 Suppl 1**, S117-121
29. Del Prato, S., Wishner, W. J., Gromada, J., and Schluchter, B. J. (2004) Beta-cell mass plasticity in type 2 diabetes. *Diabetes, obesity & metabolism* **6**, 319-331
30. Masiello, P. (2006) Animal models of type 2 diabetes with reduced pancreatic beta-cell mass. *The international journal of biochemistry & cell biology* **38**, 873-893
31. Guillausseau, P. J., Meas, T., Virally, M., Laloi-Michelin, M., Medeau, V., and Kevorkian, J. P. (2008) Abnormalities in insulin secretion in type 2 diabetes mellitus. *Diabetes & metabolism* **34 Suppl 2**, S43-48
32. Bilan, P. J., Samokhvalov, V., Koshkina, A., Schertzer, J. D., Samaan, M. C., and Klip, A. (2009) Direct and macrophage-mediated actions of fatty acids causing insulin resistance in muscle cells. *Archives of physiology and biochemistry* **115**, 176-190
33. Bonen, A., Dohm, G. L., and van Loon, L. J. (2006) Lipid metabolism, exercise and insulin action. *Essays in biochemistry* **42**, 47-59
34. Dyck, D. J., Heigenhauser, G. J., and Bruce, C. R. (2006) The role of adipokines as regulators of skeletal muscle fatty acid metabolism and insulin sensitivity. *Acta physiologica* **186**, 5-16
35. Guilherme, A., Virbasius, J. V., Puri, V., and Czech, M. P. (2008) Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nature reviews. Molecular cell biology* **9**, 367-377

36. Savage, D. B., Petersen, K. F., and Shulman, G. I. (2007) Disordered lipid metabolism and the pathogenesis of insulin resistance. *Physiological reviews* **87**, 507-520
37. Zeggini, E., Scott, L. J., Saxena, R., Voight, B. F., Marchini, J. L., Hu, T., de Bakker, P. I., Abecasis, G. R., Almgren, P., Andersen, G., Ardlie, K., Bostrom, K. B., Bergman, R. N., Bonnycastle, L. L., Borch-Johnsen, K., Burtt, N. P., Chen, H., Chines, P. S., Daly, M. J., Deodhar, P., Ding, C. J., Doney, A. S., Duren, W. L., Elliott, K. S., Erdos, M. R., Frayling, T. M., Freathy, R. M., Gianniny, L., Grallert, H., Grarup, N., Groves, C. J., Guiducci, C., Hansen, T., Herder, C., Hitman, G. A., Hughes, T. E., Isomaa, B., Jackson, A. U., Jorgensen, T., Kong, A., Kubalanza, K., Kuruvilla, F. G., Kuusisto, J., Langenberg, C., Lango, H., Lauritzen, T., Li, Y., Lindgren, C. M., Lyssenko, V., Marvelle, A. F., Meisinger, C., Midthjell, K., Mohlke, K. L., Morken, M. A., Morris, A. D., Narisu, N., Nilsson, P., Owen, K. R., Palmer, C. N., Payne, F., Perry, J. R., Pettersen, E., Platou, C., Prokopenko, I., Qi, L., Qin, L., Rayner, N. W., Rees, M., Roix, J. J., Sandbaek, A., Shields, B., Sjogren, M., Steinthorsdottir, V., Stringham, H. M., Swift, A. J., Thorleifsson, G., Thorsteinsdottir, U., Timpson, N. J., Tuomi, T., Tuomilehto, J., Walker, M., Watanabe, R. M., Weedon, M. N., Willer, C. J., Wellcome Trust Case Control, C., Illig, T., Hveem, K., Hu, F. B., Laakso, M., Stefansson, K., Pedersen, O., Wareham, N. J., Barroso, I., Hattersley, A. T., Collins, F. S., Groop, L., McCarthy, M. I., Boehnke, M., and Altshuler, D. (2008) Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nature genetics* **40**, 638-645
38. Ali, O. (2013) Genetics of type 2 diabetes. *World journal of diabetes* **4**, 114-123
39. Lau, D. C., and Teoh, H. (2013) Benefits of modest weight loss on the management of type 2 diabetes mellitus. *Canadian journal of diabetes* **37**, 128-134
40. Nyenwe, E. A., Jerkins, T. W., Umpierrez, G. E., and Kitabchi, A. E. (2011) Management of type 2 diabetes: evolving strategies for the treatment of patients with type 2 diabetes. *Metabolism: clinical and experimental* **60**, 1-23
41. Bennett, W. L., Wilson, L. M., Bolen, S., Maruthur, N., Singh, S., Chatterjee, R., Marinopoulos, S. S., Puhan, M. A., Ranasinghe, P., Nicholson, W. K., Block, L., Odelola, O., Dalal, D. S., Ogbeche, G. E., Chandrasekhar, A., Hutfless, S., Bass, E. B., and Segal, J. B. (2011). in *Oral Diabetes Medications for Adults With Type 2 Diabetes: An Update*, Rockville (MD). pp
42. Jonas, D., Van Scoyoc, E., Gerrald, K., Wines, R., Amick, H., Triplette, M., and Runge, T. (2011). in *Drug Class Review: Newer Diabetes Medications, TZDs, and Combinations: Final Original Report*, Portland (OR). pp
43. Burcelin, R., Knauf, C., and Cani, P. D. (2008) Pancreatic alpha-cell dysfunction in diabetes. *Diabetes & metabolism* **34 Suppl 2**, S49-55
44. Del Prato, S., and Marchetti, P. (2004) Beta- and alpha-cell dysfunction in type 2 diabetes. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* **36**, 775-781
45. Woods, S. C., Lutz, T. A., Geary, N., and Langhans, W. (2006) Pancreatic signals controlling food intake; insulin, glucagon and amylin. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **361**, 1219-1235
46. Friedman, J. M., and Halaas, J. L. (1998) Leptin and the regulation of body weight in mammals. *Nature* **395**, 763-770
47. Ioffe, E., Moon, B., Connolly, E., and Friedman, J. M. (1998) Abnormal regulation of the leptin gene in the pathogenesis of obesity. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 11852-11857

48. Figlewicz, D. P., Bennett, J., Evans, S. B., Kaiyala, K., Sipols, A. J., and Benoit, S. C. (2004) Intraventricular insulin and leptin reverse place preference conditioned with high-fat diet in rats. *Behavioral neuroscience* **118**, 479-487
49. Angelidis, G., Valotassiou, V., and Georgoulias, P. (2010) Current and potential roles of ghrelin in clinical practice. *Journal of endocrinological investigation* **33**, 823-838
50. Burcelin, R., Cani, P. D., and Knauf, C. (2007) Glucagon-like peptide-1 and energy homeostasis. *The Journal of nutrition* **137**, 2534S-2538S
51. Iynedjian, P. B., Pilot, P. R., Nouspikel, T., Milburn, J. L., Quaade, C., Hughes, S., Ucla, C., and Newgard, C. B. (1989) Differential expression and regulation of the glucokinase gene in liver and islets of Langerhans. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 7838-7842
52. Pozza, G., Galansino, G., Hoffeld, H., and Foa, P. P. (1958) Stimulation of insulin output by monosaccharides and monosaccharide derivatives. *The American journal of physiology* **192**, 497-500
53. Thorens, B., Sarkar, H. K., Kaback, H. R., and Lodish, H. F. (1988) Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney, and beta-pancreatic islet cells. *Cell* **55**, 281-290
54. Curry, D. L., Bennett, L. L., and Grodsky, G. M. (1968) Dynamics of insulin secretion by the perfused rat pancreas. *Endocrinology* **83**, 572-584
55. Trus, M. D., Zawalich, W. S., Burch, P. T., Berner, D. K., Weill, V. A., and Matschinsky, F. M. (1981) Regulation of glucose metabolism in pancreatic islets. *Diabetes* **30**, 911-922
56. Ashcroft, F. M., and Rorsman, P. (1989) Electrophysiology of the pancreatic beta-cell. *Progress in biophysics and molecular biology* **54**, 87-143
57. Ashcroft, F. M., Rorsman, P., and Trube, G. (1989) Single calcium channel activity in mouse pancreatic beta-cells. *Annals of the New York Academy of Sciences* **560**, 410-412
58. Henquin, J. C. (2009) Regulation of insulin secretion: a matter of phase control and amplitude modulation. *Diabetologia* **52**, 739-751
59. Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., Clement, J. P. t., Boyd, A. E., 3rd, Gonzalez, G., Herrera-Sosa, H., Nguy, K., Bryan, J., and Nelson, D. A. (1995) Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* **268**, 423-426
60. Cook, D. L., and Hales, C. N. (1984) Intracellular ATP directly blocks K+ channels in pancreatic B-cells. *Nature* **311**, 271-273
61. Straub, S. G., and Sharp, G. W. (2002) Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes/metabolism research and reviews* **18**, 451-463
62. Shiota, C., Larsson, O., Shelton, K. D., Shiota, M., Efanov, A. M., Hoy, M., Lindner, J., Kooptiwut, S., Juntti-Berggren, L., Gromada, J., Berggren, P. O., and Magnuson, M. A. (2002) Sulfonylurea receptor type 1 knock-out mice have intact feeding-stimulated insulin secretion despite marked impairment in their response to glucose. *The Journal of biological chemistry* **277**, 37176-37183
63. Szollosi, A., Nenquin, M., Aguilar-Bryan, L., Bryan, J., and Henquin, J. C. (2007) Glucose stimulates Ca2+ influx and insulin secretion in 2-week-old beta-cells lacking ATP-sensitive K+ channels. *The Journal of biological chemistry* **282**, 1747-1756
64. Gembal, M., Gilon, P., and Henquin, J. C. (1992) Evidence that glucose can control insulin release independently from its action on ATP-sensitive K+ channels in mouse B cells. *The Journal of clinical investigation* **89**, 1288-1295

65. Corkey, B. E., Deeney, J. T., Yaney, G. C., Tornheim, K., and Prentki, M. (2000) The role of long-chain fatty acyl-CoA esters in beta-cell signal transduction. *The Journal of nutrition* **130**, 299S-304S
66. Corkey, B. E., Glennon, M. C., Chen, K. S., Deeney, J. T., Matschinsky, F. M., and Prentki, M. (1989) A role for malonyl-CoA in glucose-stimulated insulin secretion from clonal pancreatic beta-cells. *The Journal of biological chemistry* **264**, 21608-21612
67. Ivarsson, R., Quintens, R., Dejonghe, S., Tsukamoto, K., in 't Veld, P., Renstrom, E., and Schuit, F. C. (2005) Redox control of exocytosis: regulatory role of NADPH, thioredoxin, and glutaredoxin. *Diabetes* **54**, 2132-2142
68. Maechler, P., Gjinovci, A., and Wollheim, C. B. (2002) Implication of glutamate in the kinetics of insulin secretion in rat and mouse perfused pancreas. *Diabetes* **51 Suppl 1**, S99-102
69. Maechler, P., and Wollheim, C. B. (2000) Mitochondrial signals in glucose-stimulated insulin secretion in the beta cell. *The Journal of physiology* **529 Pt 1**, 49-56
70. Lu, D., Mulder, H., Zhao, P., Burgess, S. C., Jensen, M. V., Kamzolova, S., Newgard, C. B., and Sherry, A. D. (2002) 13C NMR isotopomer analysis reveals a connection between pyruvate cycling and glucose-stimulated insulin secretion (GSIS). *Proceedings of the National Academy of Sciences of the United States of America* **99**, 2708-2713
71. MacDonald, M. J., Fahien, L. A., Brown, L. J., Hasan, N. M., Buss, J. D., and Kendrick, M. A. (2005) Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion. *American journal of physiology. Endocrinology and metabolism* **288**, E1-15
72. Hasan, N. M., Longacre, M. J., Stoker, S. W., Boonsaen, T., Jitrapakdee, S., Kendrick, M. A., Wallace, J. C., and MacDonald, M. J. (2008) Impaired anaplerosis and insulin secretion in insulinoma cells caused by small interfering RNA-mediated suppression of pyruvate carboxylase. *The Journal of biological chemistry* **283**, 28048-28059
73. MacDonald, M. J. (1993) Metabolism of the insulin secretagogue methyl succinate by pancreatic islets. *Archives of biochemistry and biophysics* **300**, 201-205
74. Schuit, F., De Vos, A., Farfari, S., Moens, K., Pipeleers, D., Brun, T., and Prentki, M. (1997) Metabolic fate of glucose in purified islet cells. Glucose-regulated anaplerosis in beta cells. *The Journal of biological chemistry* **272**, 18572-18579
75. Prentki, M., Joly, E., El-Assaad, W., and Roduit, R. (2002) Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes. *Diabetes* **51 Suppl 3**, S405-413
76. Joseph, J. W., Odegaard, M. L., Ronnebaum, S. M., Burgess, S. C., Muehlbauer, J., Sherry, A. D., and Newgard, C. B. (2007) Normal flux through ATP-citrate lyase or fatty acid synthase is not required for glucose-stimulated insulin secretion. *The Journal of biological chemistry* **282**, 31592-31600
77. Boucher, A., Lu, D., Burgess, S. C., Telemaque-Potts, S., Jensen, M. V., Mulder, H., Wang, M. Y., Unger, R. H., Sherry, A. D., and Newgard, C. B. (2004) Biochemical mechanism of lipid-induced impairment of glucose-stimulated insulin secretion and reversal with a malate analogue. *The Journal of biological chemistry* **279**, 27263-27271
78. Cline, G. W., Lepine, R. L., Papas, K. K., Kibbey, R. G., and Shulman, G. I. (2004) 13C NMR isotopomer analysis of anaplerotic pathways in INS-1 cells. *The Journal of biological chemistry* **279**, 44370-44375
79. Jensen, M. V., Joseph, J. W., Ronnebaum, S. M., Burgess, S. C., Sherry, A. D., and Newgard, C. B. (2008) Metabolic cycling in control of glucose-stimulated insulin secretion. *American journal of physiology. Endocrinology and metabolism* **295**, E1287-1297

80. Jensen, M. V., Joseph, J. W., Ilkayeva, O., Burgess, S., Lu, D., Ronnebaum, S. M., Odegaard, M., Becker, T. C., Sherry, A. D., and Newgard, C. B. (2006) Compensatory responses to pyruvate carboxylase suppression in islet beta-cells. Preservation of glucose-stimulated insulin secretion. *The Journal of biological chemistry* **281**, 22342-22351
81. Eto, K., Tsubamoto, Y., Terauchi, Y., Sugiyama, T., Kishimoto, T., Takahashi, N., Yamauchi, N., Kubota, N., Murayama, S., Aizawa, T., Akanuma, Y., Aizawa, S., Kasai, H., Yazaki, Y., and Kadowaki, T. (1999) Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion. *Science* **283**, 981-985
82. Rubi, B., del Arco, A., Bartley, C., Satrustegui, J., and Maechler, P. (2004) The malate-aspartate NADH shuttle member Aralar1 determines glucose metabolic fate, mitochondrial activity, and insulin secretion in beta cells. *The Journal of biological chemistry* **279**, 55659-55666
83. Lee, C. Y., Chasalow, F., Lee, S. M., Lewis, S., and Johnson, F. M. (1980) A null mutation of cytoplasmic malic enzyme in mice. *Molecular and cellular biochemistry* **30**, 143-149
84. MacDonald, M. J., and Marshall, L. K. (2001) Survey of normal appearing mouse strain which lacks malic enzyme and Nad+-linked glycerol phosphate dehydrogenase: normal pancreatic beta cell function, but abnormal metabolite pattern in skeletal muscle. *Molecular and cellular biochemistry* **220**, 117-125
85. Ronnebaum, S. M., Jensen, M. V., Hohmeier, H. E., Burgess, S. C., Zhou, Y. P., Qian, S., MacNeil, D., Howard, A., Thornberry, N., Ilkayeva, O., Lu, D., Sherry, A. D., and Newgard, C. B. (2008) Silencing of cytosolic or mitochondrial isoforms of malic enzyme has no effect on glucose-stimulated insulin secretion from rodent islets. *The Journal of biological chemistry* **283**, 28909-28917
86. Farfari, S., Schulz, V., Corkey, B., and Prentki, M. (2000) Glucose-regulated anaplerosis and cataplerosis in pancreatic beta-cells: possible implication of a pyruvate/citrate shuttle in insulin secretion. *Diabetes* **49**, 718-726
87. Guay, C., Madiraju, S. R., Aumais, A., Joly, E., and Prentki, M. (2007) A role for ATP-citrate lyase, malic enzyme, and pyruvate/citrate cycling in glucose-induced insulin secretion. *The Journal of biological chemistry* **282**, 35657-35665
88. Joseph, J. W., Jensen, M. V., Ilkayeva, O., Palmieri, F., Alarcon, C., Rhodes, C. J., and Newgard, C. B. (2006) The mitochondrial citrate/isocitrate carrier plays a regulatory role in glucose-stimulated insulin secretion. *The Journal of biological chemistry* **281**, 35624-35632
89. Flamez, D., Berger, V., Kruhoffer, M., Orntoft, T., Pipeleers, D., and Schuit, F. C. (2002) Critical role for cataplerosis via citrate in glucose-regulated insulin release. *Diabetes* **51**, 2018-2024
90. Rabaglia, M. E., Gray-Keller, M. P., Frey, B. L., Shortreed, M. R., Smith, L. M., and Attie, A. D. (2005) Alpha-Ketoisocaproate-induced hypersecretion of insulin by islets from diabetes-susceptible mice. *American journal of physiology. Endocrinology and metabolism* **289**, E218-224
91. Ronnebaum, S. M., Ilkayeva, O., Burgess, S. C., Joseph, J. W., Lu, D., Stevens, R. D., Becker, T. C., Sherry, A. D., Newgard, C. B., and Jensen, M. V. (2006) A pyruvate cycling pathway involving cytosolic NADP-dependent isocitrate dehydrogenase regulates glucose-stimulated insulin secretion. *The Journal of biological chemistry* **281**, 30593-30602
92. Liu, S. Q., Jin, H., Zacarias, A., Srivastava, S., and Bhatnagar, A. (2001) Binding of pyridine nucleotide coenzymes to the beta-subunit of the voltage-sensitive K+ channel. *The Journal of biological chemistry* **276**, 11812-11820
93. Tipparaju, S. M., Li, X. P., Kilfoil, P. J., Xue, B., Uversky, V. N., Bhatnagar, A., and Barski, O. A. (2012) Interactions between the C-terminus of Kv1.5 and Kvbeta regulate pyridine

nucleotide-dependent changes in channel gating. *Pflugers Archiv : European journal of physiology* **463**, 799-818
94. MacDonald, P. E., Salapatek, A. M., and Wheeler, M. B. (2003) Temperature and redox state dependence of native Kv2.1 currents in rat pancreatic beta-cells. *The Journal of physiology* **546**, 647-653
95. Jacobson, D. A., Kuznetsov, A., Lopez, J. P., Kash, S., Ammala, C. E., and Philipson, L. H. (2007) Kv2.1 ablation alters glucose-induced islet electrical activity, enhancing insulin secretion. *Cell metabolism* **6**, 229-235
96. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 5510-5514
97. Johnson, B., Brooks, B. A., Heinzmann, C., Diep, A., Mohandas, T., Sparkes, R. S., Reyes, H., Hoffman, E., Lange, E., Gatti, R. A., and et al. (1993) The Ah receptor nuclear translocator gene (ARNT) is located on q21 of human chromosome 1 and on mouse chromosome 3 near Cf-3. *Genomics* **17**, 592-598
98. Wang, F., Gao, J. X., Mimura, J., Kobayashi, A., Sogawa, K., and Fujii-Kuriyama, Y. (1998) Structure and expression of the mouse AhR nuclear translocator (mArnt) gene. *The Journal of biological chemistry* **273**, 24867-24873
99. Pollenz, R. S., Sattler, C. A., and Poland, A. (1994) The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. *Molecular pharmacology* **45**, 428-438
100. Abbott, B. D., Probst, M. R., and Perdew, G. H. (1994) Immunohistochemical double-staining for Ah receptor and ARNT in human embryonic palatal shelves. *Teratology* **50**, 361-366
101. Kobayashi, A., Numayama-Tsuruta, K., Sogawa, K., and Fujii-Kuriyama, Y. (1997) CBP/p300 functions as a possible transcriptional coactivator of Ah receptor nuclear translocator (Arnt). *Journal of biochemistry* **122**, 703-710
102. Wang, G. L., and Semenza, G. L. (1995) Purification and characterization of hypoxia-inducible factor 1. *The Journal of biological chemistry* **270**, 1230-1237
103. Partch, C. L., and Gardner, K. H. (2011) Coactivators necessary for transcriptional output of the hypoxia inducible factor, HIF, are directly recruited by ARNT PAS-B. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 7739-7744
104. Swanson, H. I., Chan, W. K., and Bradfield, C. A. (1995) DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. *The Journal of biological chemistry* **270**, 26292-26302
105. Jiang, B. H., Rue, E., Wang, G. L., Roe, R., and Semenza, G. L. (1996) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *The Journal of biological chemistry* **271**, 17771-17778
106. Lindebro, M. C., Poellinger, L., and Whitelaw, M. L. (1995) Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. *The EMBO journal* **14**, 3528-3539
107. Reyes, H., Reisz-Porszasz, S., and Hankinson, O. (1992) Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science* **256**, 1193-1195
108. Reisz-Porszasz, S., Probst, M. R., Fukunaga, B. N., and Hankinson, O. (1994) Identification of functional domains of the aryl hydrocarbon receptor nuclear translocator protein (ARNT). *Molecular and cellular biology* **14**, 6075-6086

109. Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., and Bradfield, C. A. (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* **103**, 1009-1017
110. Hogenesch, J. B., Chan, W. K., Jackiw, V. H., Brown, R. C., Gu, Y. Z., Pray-Grant, M., Perdew, G. H., and Bradfield, C. A. (1997) Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *The Journal of biological chemistry* **272**, 8581-8593
111. Ikeda, M., and Nomura, M. (1997) cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS protein (BMAL1) and identification of alternatively spliced variants with alternative translation initiation site usage. *Biochemical and biophysical research communications* **233**, 258-264
112. Hirose, K., Morita, M., Ema, M., Mimura, J., Hamada, H., Fujii, H., Saijo, Y., Gotoh, O., Sogawa, K., and Fujii-Kuriyama, Y. (1996) cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS factor (Arnt2) with close sequence similarity to the aryl hydrocarbon receptor nuclear translocator (Arnt). *Molecular and cellular biology* **16**, 1706-1713
113. Drutel, G., Kathmann, M., Heron, A., Schwartz, J. C., and Arrang, J. M. (1996) Cloning and selective expression in brain and kidney of ARNT2 homologous to the Ah receptor nuclear translocator (ARNT). *Biochemical and biophysical research communications* **225**, 333-339
114. Korkalainen, M., Tuomisto, J., and Pohjanvirta, R. (2003) Identification of novel splice variants of ARNT and ARNT2 in the rat. *Biochemical and biophysical research communications* **303**, 1095-1100
115. Furness, S. G., Lees, M. J., and Whitelaw, M. L. (2007) The dioxin (aryl hydrocarbon) receptor as a model for adaptive responses of bHLH/PAS transcription factors. *FEBS letters* **581**, 3616-3625
116. Kewley, R. J., Whitelaw, M. L., and Chapman-Smith, A. (2004) The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *The international journal of biochemistry & cell biology* **36**, 189-204
117. McIntosh, B. E., Hogenesch, J. B., and Bradfield, C. A. (2010) Mammalian Per-Arnt-Sim proteins in environmental adaptation. *Annual review of physiology* **72**, 625-645
118. Antonsson, C., Arulampalam, V., Whitelaw, M. L., Pettersson, S., and Poellinger, L. (1995) Constitutive function of the basic helix-loop-helix/PAS factor Arnt. Regulation of target promoters via the E box motif. *The Journal of biological chemistry* **270**, 13968-13972
119. Sogawa, K., Nakano, R., Kobayashi, A., Kikuchi, Y., Ohe, N., Matsushita, N., and Fujii-Kuriyama, Y. (1995) Possible function of Ah receptor nuclear translocator (Arnt) homodimer in transcriptional regulation. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 1936-1940
120. Arpiainen, S., Lamsa, V., Pelkonen, O., Yim, S. H., Gonzalez, F. J., and Hakkola, J. (2007) Aryl hydrocarbon receptor nuclear translocator and upstream stimulatory factor regulate Cytochrome P450 2a5 transcription through a common E-box site. *Journal of molecular biology* **369**, 640-652
121. Wolff, M., Jelkmann, W., Dunst, J., and Depping, R. (2013) The Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT/HIF-1beta) is Influenced by Hypoxia and Hypoxia-Mimetics. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* **32**, 849-858
122. Li, H., Ko, H. P., and Whitlock, J. P. (1996) Induction of phosphoglycerate kinase 1 gene expression by hypoxia. Roles of Arnt and HIF1alpha. *The Journal of biological chemistry* **271**, 21262-21267

123. Salceda, S., Beck, I., and Caro, J. (1996) Absolute requirement of aryl hydrocarbon receptor nuclear translocator protein for gene activation by hypoxia. *Archives of biochemistry and biophysics* **334**, 389-394
124. Kallio, P. J., Pongratz, I., Gradin, K., McGuire, J., and Poellinger, L. (1997) Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 5667-5672
125. Piret, J. P., Mottet, D., Raes, M., and Michiels, C. (2002) CoCl2, a chemical inducer of hypoxia-inducible factor-1, and hypoxia reduce apoptotic cell death in hepatoma cell line HepG2. *Annals of the New York Academy of Sciences* **973**, 443-447
126. Choi, K. S., Park, S. Y., Baek, S. H., Dey-Rao, R., Park, Y. M., Zhang, H., Ip, C., Park, E. M., Kim, Y. H., and Park, J. H. (2006) Analysis of protein redox modification by hypoxia. *Preparative biochemistry & biotechnology* **36**, 65-79
127. Choi, S. M., Oh, H., and Park, H. (2008) Microarray analyses of hypoxia-regulated genes in an aryl hydrocarbon receptor nuclear translocator (Arnt)-dependent manner. *The FEBS journal* **275**, 5618-5634
128. Pongratz, I., Stromstedt, P. E., Mason, G. G., and Poellinger, L. (1991) Inhibition of the specific DNA binding activity of the dioxin receptor by phosphatase treatment. *The Journal of biological chemistry* **266**, 16813-16817
129. Berghard, A., Gradin, K., Pongratz, I., Whitelaw, M., and Poellinger, L. (1993) Cross-coupling of signal transduction pathways: the dioxin receptor mediates induction of cytochrome P-450IA1 expression via a protein kinase C-dependent mechanism. *Molecular and cellular biology* **13**, 677-689
130. Tsai, J. C., and Perdew, G. H. (1997) Ah receptor nuclear translocator protein heterogeneity is altered after heterodimerization with the Ah receptor. *Biochemistry* **36**, 9066-9072
131. Levine, S. L., and Perdew, G. H. (2001) Aryl hydrocarbon receptor (AhR)/AhR nuclear translocator (ARNT) activity is unaltered by phosphorylation of a periodicity/ARNT/single-minded (PAS)-region serine residue. *Molecular pharmacology* **59**, 557-566
132. Swanson, H. I. (2002) DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation. *Chemico-biological interactions* **141**, 63-76
133. Beischlag, T. V., Luis Morales, J., Hollingshead, B. D., and Perdew, G. H. (2008) The aryl hydrocarbon receptor complex and the control of gene expression. *Critical reviews in eukaryotic gene expression* **18**, 207-250
134. Sogawa, K., and Fujii-Kuriyama, Y. (1997) Ah receptor, a novel ligand-activated transcription factor. *Journal of biochemistry* **122**, 1075-1079
135. Perdew, G. H. (1988) Association of the Ah receptor with the 90-kDa heat shock protein. *The Journal of biological chemistry* **263**, 13802-13805
136. Whitelaw, M. L., Gottlicher, M., Gustafsson, J. A., and Poellinger, L. (1993) Definition of a novel ligand binding domain of a nuclear bHLH receptor: co-localization of ligand and hsp90 binding activities within the regulable inactivation domain of the dioxin receptor. *The EMBO journal* **12**, 4169-4179
137. Probst, M. R., Reisz-Porszasz, S., Agbunag, R. V., Ong, M. S., and Hankinson, O. (1993) Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action. *Molecular pharmacology* **44**, 511-518
138. Li, H., Dong, L., and Whitlock, J. P., Jr. (1994) Transcriptional activation function of the mouse Ah receptor nuclear translocator. *The Journal of biological chemistry* **269**, 28098-28105

139. Lim, W., Park, Y., Cho, J., Park, C., Park, J., Park, Y. K., Park, H., and Lee, Y. (2011) Estrogen receptor beta inhibits transcriptional activity of hypoxia inducible factor-1 through the downregulation of arylhydrocarbon receptor nuclear translocator. *Breast cancer research : BCR* **13**, R32
140. Ruegg, J., Swedenborg, E., Wahlstrom, D., Escande, A., Balaguer, P., Pettersson, K., and Pongratz, I. (2008) The transcription factor aryl hydrocarbon receptor nuclear translocator functions as an estrogen receptor beta-selective coactivator, and its recruitment to alternative pathways mediates antiestrogenic effects of dioxin. *Molecular endocrinology* **22**, 304-316
141. Semenza, G. L., and Wang, G. L. (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and cellular biology* **12**, 5447-5454
142. Ke, Q., and Costa, M. (2006) Hypoxia-inducible factor-1 (HIF-1). *Molecular pharmacology* **70**, 1469-1480
143. Schofield, C. J., and Ratcliffe, P. J. (2004) Oxygen sensing by HIF hydroxylases. *Nature reviews. Molecular cell biology* **5**, 343-354
144. Kaelin, W. G., Jr., and Ratcliffe, P. J. (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Molecular cell* **30**, 393-402
145. Gassmann, M., Kvietikova, I., Rolfs, A., and Wenger, R. H. (1997) Oxygen- and dioxin-regulated gene expression in mouse hepatoma cells. *Kidney international* **51**, 567-574
146. Wood, S. M., Gleadle, J. M., Pugh, C. W., Hankinson, O., and Ratcliffe, P. J. (1996) The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. *The Journal of biological chemistry* **271**, 15117-15123
147. Brahimi-Horn, C., and Pouyssegur, J. (2006) The role of the hypoxia-inducible factor in tumor metabolism growth and invasion. *Bulletin du cancer* **93**, E73-80
148. Buchler, P., Reber, H. A., Buchler, M., Shrinkante, S., Buchler, M. W., Friess, H., Semenza, G. L., and Hines, O. J. (2003) Hypoxia-inducible factor 1 regulates vascular endothelial growth factor expression in human pancreatic cancer. *Pancreas* **26**, 56-64
149. Bunn, H. F., Gu, J., Huang, L. E., Park, J. W., and Zhu, H. (1998) Erythropoietin: a model system for studying oxygen-dependent gene regulation. *The Journal of experimental biology* **201**, 1197-1201
150. Wang, F., Shi, S., Zhang, R., and Hankinson, O. (2006) Identifying target genes of the aryl hydrocarbon receptor nuclear translocator (Arnt) using DNA microarray analysis. *Biological chemistry* **387**, 1215-1218
151. Wenger, R. H., and Gassmann, M. (1997) Oxygen(es) and the hypoxia-inducible factor-1. *Biological chemistry* **378**, 609-616
152. Simon, M. C., and Keith, B. (2008) The role of oxygen availability in embryonic development and stem cell function. *Nature reviews. Molecular cell biology* **9**, 285-296
153. Sainson, R. C., and Harris, A. L. (2006) Hypoxia-regulated differentiation: let's step it up a Notch. *Trends in molecular medicine* **12**, 141-143
154. Semenza, G. L. (1994) Transcriptional regulation of gene expression: mechanisms and pathophysiology. *Human mutation* **3**, 180-199
155. Brahimi-Horn, M. C., and Pouyssegur, J. (2009) HIF at a glance. *Journal of cell science* **122**, 1055-1057
156. Gorlach, A., and Bonello, S. (2008) The cross-talk between NF-kappaB and HIF-1: further evidence for a significant liaison. *The Biochemical journal* **412**, e17-19

157. Rius, J., Guma, M., Schachtrup, C., Akassoglou, K., Zinkernagel, A. S., Nizet, V., Johnson, R. S., Haddad, G. G., and Karin, M. (2008) NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature* **453**, 807-811
158. van Uden, P., Kenneth, N. S., and Rocha, S. (2008) Regulation of hypoxia-inducible factor-1alpha by NF-kappaB. *The Biochemical journal* **412**, 477-484
159. Fedele, A. O., Whitelaw, M. L., and Peet, D. J. (2002) Regulation of gene expression by the hypoxia-inducible factors. *Molecular interventions* **2**, 229-243
160. Carmeliet, P., Ng, Y. S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V. V., Stalmans, I., Mattot, V., Perriard, J. C., Dewerchin, M., Flameng, W., Nagy, A., Lupu, F., Moons, L., Collen, D., D'Amore, P. A., and Shima, D. T. (1999) Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Nature medicine* **5**, 495-502
161. Maltepe, E., Schmidt, J. V., Baunoch, D., Bradfield, C. A., and Simon, M. C. (1997) Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature* **386**, 403-407
162. Kozak, K. R., Abbott, B., and Hankinson, O. (1997) ARNT-deficient mice and placental differentiation. *Developmental biology* **191**, 297-305
163. Tomita, S., Sinal, C. J., Yim, S. H., and Gonzalez, F. J. (2000) Conditional disruption of the aryl hydrocarbon receptor nuclear translocator (Arnt) gene leads to loss of target gene induction by the aryl hydrocarbon receptor and hypoxia-inducible factor 1alpha. *Molecular endocrinology* **14**, 1674-1681
164. Gunton, J. E., Kulkarni, R. N., Yim, S., Okada, T., Hawthorne, W. J., Tseng, Y. H., Roberson, R. S., Ricordi, C., O'Connell, P. J., Gonzalez, F. J., and Kahn, C. R. (2005) Loss of ARNT/HIF1beta mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell* **122**, 337-349
165. Tsuchiya, T., Kominato, Y., and Ueda, M. (2002) Human hypoxic signal transduction through a signature motif in hepatocyte nuclear factor 4. *Journal of biochemistry* **132**, 37-44
166. Zhang, H., Sun, S., Herreman, A., De Strooper, B., and Bezprozvanny, I. (2010) Role of presenilins in neuronal calcium homeostasis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**, 8566-8580
167. Ghidoni, R., Gasparini, L., Alberici, A., Benussi, L., Barbiero, L., Mazzoli, F., Nicosia, F., Finazzi, D., Benerini Gatta, L., Albertini, A., Zanetti, O., and Binetti, G. (2003) Inhibition of energy metabolism down-regulates the Alzheimer related presenilin 2 gene. *Journal of neural transmission* **110**, 1029-1039
168. Dror, V., Kalynyak, T. B., Bychkivska, Y., Frey, M. H., Tee, M., Jeffrey, K. D., Nguyen, V., Luciani, D. S., and Johnson, J. D. (2008) Glucose and endoplasmic reticulum calcium channels regulate HIF-1beta via presenilin in pancreatic beta-cells. *The Journal of biological chemistry* **283**, 9909-9916
169. Noordeen, N. A., Khera, T. K., Sun, G., Longbottom, E. R., Pullen, T. J., da Silva Xavier, G., Rutter, G. A., and Leclerc, I. (2010) Carbohydrate-responsive element-binding protein (ChREBP) is a negative regulator of ARNT/HIF-1beta gene expression in pancreatic islet beta-cells. *Diabetes* **59**, 153-160
170. Cheng, K., Ho, K., Stokes, R., Scott, C., Lau, S. M., Hawthorne, W. J., O'Connell, P. J., Loudovaris, T., Kay, T. W., Kulkarni, R. N., Okada, T., Wang, X. L., Yim, S. H., Shah, Y., Grey, S. T., Biankin, A. V., Kench, J. G., Laybutt, D. R., Gonzalez, F. J., Kahn, C. R., and Gunton, J. E. (2010) Hypoxia-inducible factor-1alpha regulates beta cell function in mouse and human islets. *The Journal of clinical investigation* **120**, 2171-2183

171. Michael, M. D., Kulkarni, R. N., Postic, C., Previs, S. F., Shulman, G. I., Magnuson, M. A., and Kahn, C. R. (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Molecular cell* **6**, 87-97
172. Saltiel, A. R., and Kahn, C. R. (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**, 799-806
173. Wang, X. L., Suzuki, R., Lee, K., Tran, T., Gunton, J. E., Saha, A. K., Patti, M. E., Goldfine, A., Ruderman, N. B., Gonzalez, F. J., and Kahn, C. R. (2009) Ablation of ARNT/HIF1beta in liver alters gluconeogenesis, lipogenic gene expression, and serum ketones. *Cell metabolism* **9**, 428-439
174. Pedersen, T. A., Bereshchenko, O., Garcia-Silva, S., Ermakova, O., Kurz, E., Mandrup, S., Porse, B. T., and Nerlov, C. (2007) Distinct C/EBPalpha motifs regulate lipogenic and gluconeogenic gene expression in vivo. *The EMBO journal* **26**, 1081-1093
175. Qiao, L., MacLean, P. S., You, H., Schaack, J., and Shao, J. (2006) knocking down liver ccaat/enhancer-binding protein alpha by adenovirus-transduced silent interfering ribonucleic acid improves hepatic gluconeogenesis and lipid homeostasis in db/db mice. *Endocrinology* **147**, 3060-3069
176. Horton, J. D. (2002) Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. *Biochemical Society transactions* **30**, 1091-1095
177. Fantuzzi, G. (2005) Adipose tissue, adipokines, and inflammation. *The Journal of allergy and clinical immunology* **115**, 911-919; quiz 920
178. Hajer, G. R., van Haeften, T. W., and Visseren, F. L. (2008) Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *European heart journal* **29**, 2959-2971
179. Wang, B., Wood, I. S., and Trayhurn, P. (2007) Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. *Pflugers Archiv : European journal of physiology* **455**, 479-492
180. Rausch, M. E., Weisberg, S., Vardhana, P., and Tortoriello, D. V. (2008) Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration. *International journal of obesity* **32**, 451-463
181. Ye, J., Gao, Z., Yin, J., and He, Q. (2007) Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *American journal of physiology. Endocrinology and metabolism* **293**, E1118-1128
182. Lee, K. Y., Gesta, S., Boucher, J., Wang, X. L., and Kahn, C. R. (2011) The differential role of Hif1beta/Arnt and the hypoxic response in adipose function, fibrosis, and inflammation. *Cell metabolism* **14**, 491-503
183. Jiang, C., Qu, A., Matsubara, T., Chanturiya, T., Jou, W., Gavrilova, O., Shah, Y. M., and Gonzalez, F. J. (2011) Disruption of hypoxia-inducible factor 1 in adipocytes improves insulin sensitivity and decreases adiposity in high-fat diet-fed mice. *Diabetes* **60**, 2484-2495
184. Zhang, X., Lam, K. S., Ye, H., Chung, S. K., Zhou, M., Wang, Y., and Xu, A. (2010) Adipose tissue-specific inhibition of hypoxia-inducible factor 1{alpha} induces obesity and glucose intolerance by impeding energy expenditure in mice. *The Journal of biological chemistry* **285**, 32869-32877
185. Zhang, N., Fu, Z., Linke, S., Chicher, J., Gorman, J. J., Visk, D., Haddad, G. G., Poellinger, L., Peet, D. J., Powell, F., and Johnson, R. S. (2010) The asparaginyl hydroxylase factor inhibiting HIF-1alpha is an essential regulator of metabolism. *Cell metabolism* **11**, 364-378
186. Hohmeier, H. E., Mulder, H., Chen, G., Henkel-Rieger, R., Prentki, M., and Newgard, C. B. (2000) Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* **49**, 424-430

187. Perilhou, A., Tourrel-Cuzin, C., Kharroubi, I., Henique, C., Fauveau, V., Kitamura, T., Magnan, C., Postic, C., Prip-Buus, C., and Vasseur-Cognet, M. (2008) The transcription factor COUP-TFII is negatively regulated by insulin and glucose via Foxo1- and ChREBP-controlled pathways. *Molecular and cellular biology* **28**, 6568-6579
188. Roche, E., Buteau, J., Aniento, I., Reig, J. A., Soria, B., and Prentki, M. (1999) Palmitate and oleate induce the immediate-early response genes c-fos and nur-77 in the pancreatic beta-cell line INS-1. *Diabetes* **48**, 2007-2014
189. Ashcroft, S. J., Weerasinghe, L. C., Bassett, J. M., and Randle, P. J. (1972) The pentose cycle and insulin release in mouse pancreatic islets. *The Biochemical journal* **126**, 525-532
190. Detimary, P., Van den Berghe, G., and Henquin, J. C. (1996) Concentration dependence and time course of the effects of glucose on adenine and guanine nucleotides in mouse pancreatic islets. *The Journal of biological chemistry* **271**, 20559-20565
191. Fernandez, C., Fransson, U., Hallgard, E., Spegel, P., Holm, C., Krogh, M., Warell, K., James, P., and Mulder, H. (2008) Metabolomic and proteomic analysis of a clonal insulin-producing beta-cell line (INS-1 832/13). *Journal of proteome research* **7**, 400-411
192. Ray, M. K., Fagan, S. P., Moldovan, S., DeMayo, F. J., and Brunicardi, F. C. (1998) A mouse model for beta cell-specific ablation of target gene(s) using the Cre-loxP system. *Biochemical and biophysical research communications* **253**, 65-69
193. Zheng, B., Sage, M., Sheppeard, E. A., Jurecic, V., and Bradley, A. (2000) Engineering mouse chromosomes with Cre-loxP: range, efficiency, and somatic applications. *Molecular and cellular biology* **20**, 648-655
194. Kim, J. Y., van de Wall, E., Laplante, M., Azzara, A., Trujillo, M. E., Hofmann, S. M., Schraw, T., Durand, J. L., Li, H., Li, G., Jelicks, L. A., Mehler, M. F., Hui, D. Y., Deshaies, Y., Shulman, G. I., Schwartz, G. J., and Scherer, P. E. (2007) Obesity-associated improvements in metabolic profile through expansion of adipose tissue. *The Journal of clinical investigation* **117**, 2621-2637
195. Lamontagne, J., Pepin, E., Peyot, M. L., Joly, E., Ruderman, N. B., Poitout, V., Madiraju, S. R., Nolan, C. J., and Prentki, M. (2009) Pioglitazone acutely reduces insulin secretion and causes metabolic deceleration of the pancreatic beta-cell at submaximal glucose concentrations. *Endocrinology* **150**, 3465-3474
196. Malmgren, S., Nicholls, D. G., Taneera, J., Bacos, K., Koeck, T., Tamaddon, A., Wibom, R., Groop, L., Ling, C., Mulder, H., and Sharoyko, V. V. (2009) Tight coupling between glucose and mitochondrial metabolism in clonal beta-cells is required for robust insulin secretion. *The Journal of biological chemistry* **284**, 32395-32404
197. Rogers, G. W., Brand, M. D., Petrosyan, S., Ashok, D., Elorza, A. A., Ferrick, D. A., and Murphy, A. N. (2011) High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. *PloS one* **6**, e21746
198. Huijing, F., and Slater, E. C. (1961) The use of oligomycin as an inhibitor of oxidative phosphorylation. *Journal of biochemistry* **49**, 493-501
199. Allister, E. M., Robson-Doucette, C. A., Prentice, K. J., Hardy, A. B., Sultan, S., Gaisano, H. Y., Kong, D., Gilon, P., Herrera, P. L., Lowell, B. B., and Wheeler, M. B. (2013) UCP2 regulates the glucagon response to fasting and starvation. *Diabetes* **62**, 1623-1633
200. Prentki, M., and Matschinsky, F. M. (1987) Ca2+, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion. *Physiological reviews* **67**, 1185-1248
201. Henquin, J. C., Nenquin, M., Ravier, M. A., and Szollosi, A. (2009) Shortcomings of current models of glucose-induced insulin secretion. *Diabetes, obesity & metabolism* **11 Suppl 4**, 168-179

202. Ravier, M. A., Nenquin, M., Miki, T., Seino, S., and Henquin, J. C. (2009) Glucose controls cytosolic Ca2+ and insulin secretion in mouse islets lacking adenosine triphosphate-sensitive K+ channels owing to a knockout of the pore-forming subunit Kir6.2. *Endocrinology* **150**, 33-45
203. Maechler, P., Antinozzi, P. A., and Wollheim, C. B. (2000) Modulation of glutamate generation in mitochondria affects hormone secretion in INS-1E beta cells. *IUBMB life* **50**, 27-31
204. Newgard, C. B., and McGarry, J. D. (1995) Metabolic coupling factors in pancreatic beta-cell signal transduction. *Annual review of biochemistry* **64**, 689-719
205. Guo, S., Dai, C., Guo, M., Taylor, B., Harmon, J. S., Sander, M., Robertson, R. P., Powers, A. C., and Stein, R. (2013) Inactivation of specific beta cell transcription factors in type 2 diabetes. *The Journal of clinical investigation*
206. Kitamura, T. (2013) The role of FOXO1 in beta-cell failure and type 2 diabetes mellitus. *Nature reviews. Endocrinology* **9**, 615-623
207. Shao, S., Fang, Z., Yu, X., and Zhang, M. (2009) Transcription factors involved in glucose-stimulated insulin secretion of pancreatic beta cells. *Biochemical and biophysical research communications* **384**, 401-404
208. Talchai, C., Xuan, S., Lin, H. V., Sussel, L., and Accili, D. (2012) Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. *Cell* **150**, 1223-1234
209. Taylor, B. L., Liu, F. F., and Sander, M. (2013) Nkx6.1 is essential for maintaining the functional state of pancreatic Beta cells. *Cell reports* **4**, 1262-1275
210. Ochiai, D., Goda, N., Hishiki, T., Kanai, M., Senoo-Matsuda, N., Soga, T., Johnson, R. S., Yoshimura, Y., and Suematsu, M. (2011) Disruption of HIF-1alpha in hepatocytes impairs glucose metabolism in diet-induced obesity mice. *Biochemical and biophysical research communications* **415**, 445-449
211. Wang, H., Maechler, P., Antinozzi, P. A., Hagenfeldt, K. A., and Wollheim, C. B. (2000) Hepatocyte nuclear factor 4alpha regulates the expression of pancreatic beta -cell genes implicated in glucose metabolism and nutrient-induced insulin secretion. *The Journal of biological chemistry* **275**, 35953-35959
212. Miura, A., Yamagata, K., Kakei, M., Hatakeyama, H., Takahashi, N., Fukui, K., Nammo, T., Yoneda, K., Inoue, Y., Sladek, F. M., Magnuson, M. A., Kasai, H., Miyagawa, J., Gonzalez, F. J., and Shimomura, I. (2006) Hepatocyte nuclear factor-4alpha is essential for glucose-stimulated insulin secretion by pancreatic beta-cells. *The Journal of biological chemistry* **281**, 5246-5257
213. Gupta, R. K., Vatamaniuk, M. Z., Lee, C. S., Flaschen, R. C., Fulmer, J. T., Matschinsky, F. M., Duncan, S. A., and Kaestner, K. H. (2005) The MODY1 gene HNF-4alpha regulates selected genes involved in insulin secretion. *The Journal of clinical investigation* **115**, 1006-1015
214. Fajans, S. S., Bell, G. I., and Polonsky, K. S. (2001) Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. *The New England journal of medicine* **345**, 971-980
215. Wang, H., Antinozzi, P. A., Hagenfeldt, K. A., Maechler, P., and Wollheim, C. B. (2000) Molecular targets of a human HNF1 alpha mutation responsible for pancreatic beta-cell dysfunction. *The EMBO journal* **19**, 4257-4264
216. Hussain, K. (2010) Mutations in pancreatic ss-cell Glucokinase as a cause of hyperinsulinaemic hypoglycaemia and neonatal diabetes mellitus. *Reviews in endocrine & metabolic disorders* **11**, 179-183

217. Habener, J. F., and Stoffers, D. A. (1998) A newly discovered role of transcription factors involved in pancreas development and the pathogenesis of diabetes mellitus. *Proceedings of the Association of American Physicians* **110**, 12-21
218. Jensen, M. V., Haldeman, J. M., Zhang, H., Lu, D., Huising, M. O., Vale, W. W., Hohmeier, H. E., Rosenberg, P., and Newgard, C. B. (2013) Control of voltage-gated potassium channel Kv2.2 expression by pyruvate-isocitrate cycling regulates glucose-stimulated insulin secretion. *The Journal of biological chemistry* **288**, 23128-23140
219. Deeney, J. T., Tornheim, K., Korchak, H. M., Prentki, M., and Corkey, B. E. (1992) Acyl-CoA esters modulate intracellular Ca2+ handling by permeabilized clonal pancreatic beta-cells. *The Journal of biological chemistry* **267**, 19840-19845
220. Prentki, M., Vischer, S., Glennon, M. C., Regazzi, R., Deeney, J. T., and Corkey, B. E. (1992) Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. *The Journal of biological chemistry* **267**, 5802-5810
221. Nolan, C. J., Madiraju, M. S., Delghingaro-Augusto, V., Peyot, M. L., and Prentki, M. (2006) Fatty acid signaling in the beta-cell and insulin secretion. *Diabetes* **55 Suppl 2**, S16-23
222. Rebelato, E., Abdulkader, F., Curi, R., and Carpinelli, A. R. (2011) Control of the intracellular redox state by glucose participates in the insulin secretion mechanism. *PloS one* **6**, e24507
223. Spegel, P., Sharoyko, V. V., Goehring, I., Danielsson, A. P., Malmgren, S., Nagorny, C. L., Andersson, L. E., Koeck, T., Sharp, G. W., Straub, S. G., Wollheim, C. B., and Mulder, H. (2013) Time-resolved metabolomics analysis of beta-cells implicates the pentose phosphate pathway in the control of insulin release. *The Biochemical journal* **450**, 595-605
224. Huypens, P., Pillai, R., Sheinin, T., Schaefer, S., Huang, M., Odegaard, M. L., Ronnebaum, S. M., Wettig, S. D., and Joseph, J. W. (2011) The dicarboxylate carrier plays a role in mitochondrial malate transport and in the regulation of glucose-stimulated insulin secretion from rat pancreatic beta cells. *Diabetologia* **54**, 135-145
225. Odegaard, M. L., Joseph, J. W., Jensen, M. V., Lu, D., Ilkayeva, O., Ronnebaum, S. M., Becker, T. C., and Newgard, C. B. (2010) The mitochondrial 2-oxoglutarate carrier is part of a metabolic pathway that mediates glucose- and glutamine-stimulated insulin secretion. *The Journal of biological chemistry* **285**, 16530-16537
226. Pongratz, R. L., Kibbey, R. G., Shulman, G. I., and Cline, G. W. (2007) Cytosolic and mitochondrial malic enzyme isoforms differentially control insulin secretion. *The Journal of biological chemistry* **282**, 200-207
227. Gao, Z., Young, R. A., Li, G., Najafi, H., Buettger, C., Sukumvanich, S. S., Wong, R. K., Wolf, B. A., and Matschinsky, F. M. (2003) Distinguishing features of leucine and alpha-ketoisocaproate sensing in pancreatic beta-cells. *Endocrinology* **144**, 1949-1957
228. Li, C., Buettger, C., Kwagh, J., Matter, A., Daikhin, Y., Nissim, I. B., Collins, H. W., Yudkoff, M., Stanley, C. A., and Matschinsky, F. M. (2004) A signaling role of glutamine in insulin secretion. *The Journal of biological chemistry* **279**, 13393-13401
229. Newsholme, P., Bender, K., Kiely, A., and Brennan, L. (2007) Amino acid metabolism, insulin secretion and diabetes. *Biochemical Society transactions* **35**, 1180-1186
230. Jhala, U. S., Canettieri, G., Screaton, R. A., Kulkarni, R. N., Krajewski, S., Reed, J., Walker, J., Lin, X., White, M., and Montminy, M. (2003) cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. *Genes & development* **17**, 1575-1580
231. Yusta, B., Baggio, L. L., Estall, J. L., Koehler, J. A., Holland, D. P., Li, H., Pipeleers, D., Ling, Z., and Drucker, D. J. (2006) GLP-1 receptor activation improves beta cell function and survival following induction of endoplasmic reticulum stress. *Cell metabolism* **4**, 391-406

232. Van de Velde, S., Hogan, M. F., and Montminy, M. (2011) mTOR links incretin signaling to HIF induction in pancreatic beta cells. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 16876-16882
233. Cornu, M., Modi, H., Kawamori, D., Kulkarni, R. N., Joffraud, M., and Thorens, B. (2010) Glucagon-like peptide-1 increases beta-cell glucose competence and proliferation by translational induction of insulin-like growth factor-1 receptor expression. *The Journal of biological chemistry* **285**, 10538-10545
234. Parker, S. M., Moore, P. C., Johnson, L. M., and Poitout, V. (2003) Palmitate potentiation of glucose-induced insulin release: a study using 2-bromopalmitate. *Metabolism: clinical and experimental* **52**, 1367-1371
235. Poitout, V. (2003) The ins and outs of fatty acids on the pancreatic beta cell. *Trends in endocrinology and metabolism: TEM* **14**, 201-203
236. Best, L., Jarman, E., and Brown, P. D. (2011) A dual action of saturated fatty acids on electrical activity in rat pancreatic beta-cells. Role of volume-regulated anion channel and KATP channel currents. *The Journal of physiology* **589**, 1307-1316
237. Remizov, O., Jakubov, R., Dufer, M., Krippeit Drews, P., Drews, G., Waring, M., Brabant, G., Wienbergen, A., Rustenbeck, I., and Schofl, C. (2003) Palmitate-induced Ca2+-signaling in pancreatic beta-cells. *Molecular and cellular endocrinology* **212**, 1-9
238. Warnotte, C., Gilon, P., Nenquin, M., and Henquin, J. C. (1994) Mechanisms of the stimulation of insulin release by saturated fatty acids. A study of palmitate effects in mouse beta-cells. *Diabetes* **43**, 703-711
239. Latour, M. G., Alquier, T., Oseid, E., Tremblay, C., Jetton, T. L., Luo, J., Lin, D. C., and Poitout, V. (2007) GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo. *Diabetes* **56**, 1087-1094
240. Cline, G. W. (2011) Fuel-Stimulated Insulin Secretion Depends upon Mitochondria Activation and the Integration of Mitochondrial and Cytosolic Substrate Cycles. *Diabetes & metabolism journal* **35**, 458-465
241. Cline, G. W., Pongratz, R. L., Zhao, X., and Papas, K. K. (2011) Rates of insulin secretion in INS-1 cells are enhanced by coupling to anaplerosis and Kreb's cycle flux independent of ATP synthesis. *Biochemical and biophysical research communications* **415**, 30-35
242. Jitrapakdee, S., Wutthisathapornchai, A., Wallace, J. C., and MacDonald, M. J. (2010) Regulation of insulin secretion: role of mitochondrial signalling. *Diabetologia* **53**, 1019-1032
243. Lorenz, M. A., El Azzouny, M. A., Kennedy, R. T., and Burant, C. F. (2013) Metabolome response to glucose in the beta-cell line INS-1 832/13. *The Journal of biological chemistry* **288**, 10923-10935
244. Stark, R., Pasquel, F., Turcu, A., Pongratz, R. L., Roden, M., Cline, G. W., Shulman, G. I., and Kibbey, R. G. (2009) Phosphoenolpyruvate cycling via mitochondrial phosphoenolpyruvate carboxykinase links anaplerosis and mitochondrial GTP with insulin secretion. *The Journal of biological chemistry* **284**, 26578-26590
245. Heart, E., Cline, G. W., Collis, L. P., Pongratz, R. L., Gray, J. P., and Smith, P. J. (2009) Role for malic enzyme, pyruvate carboxylation, and mitochondrial malate import in glucose-stimulated insulin secretion. *American journal of physiology. Endocrinology and metabolism* **296**, E1354-1362
246. Xu, J., Han, J., Long, Y. S., Epstein, P. N., and Liu, Y. Q. (2008) The role of pyruvate carboxylase in insulin secretion and proliferation in rat pancreatic beta cells. *Diabetologia* **51**, 2022-2030
247. Newsholme, P., Brennan, L., Rubi, B., and Maechler, P. (2005) New insights into amino acid metabolism, beta-cell function and diabetes. *Clinical science* **108**, 185-194

248. Giroix, M. H., Saulnier, C., and Portha, B. (1999) Decreased pancreatic islet response to L-leucine in the spontaneously diabetic GK rat: enzymatic, metabolic and secretory data. *Diabetologia* **42**, 965-977
249. Pillai, R., Huypens, P., Huang, M., Schaefer, S., Sheinin, T., Wettig, S. D., and Joseph, J. W. (2011) Aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor-1{beta} plays a critical role in maintaining glucose-stimulated anaplerosis and insulin release from pancreatic {beta}-cells. *The Journal of biological chemistry* **286**, 1014-1024
250. Lee, J. Y., Ristow, M., Lin, X., White, M. F., Magnuson, M. A., and Hennighausen, L. (2006) RIP-Cre revisited, evidence for impairments of pancreatic beta-cell function. *The Journal of biological chemistry* **281**, 2649-2653
251. Galgani, J. E., Heilbronn, L. K., Azuma, K., Kelley, D. E., Albu, J. B., Pi-Sunyer, X., Smith, S. R., Ravussin, E., and Look, A. A. R. G. (2008) Metabolic flexibility in response to glucose is not impaired in people with type 2 diabetes after controlling for glucose disposal rate. *Diabetes* **57**, 841-845
252. Brand, M. D., and Nicholls, D. G. (2011) Assessing mitochondrial dysfunction in cells. *The Biochemical journal* **435**, 297-312
253. Hedeskov, C. J., Capito, K., and Thams, P. (1987) Cytosolic ratios of free [NADPH]/[NADP+] and [NADH]/[NAD+] in mouse pancreatic islets, and nutrient-induced insulin secretion. *The Biochemical journal* **241**, 161-167
254. Pralong, W. F., Bartley, C., and Wollheim, C. B. (1990) Single islet beta-cell stimulation by nutrients: relationship between pyridine nucleotides, cytosolic Ca2+ and secretion. *The EMBO journal* **9**, 53-60
255. Denton, R. M. (2009) Regulation of mitochondrial dehydrogenases by calcium ions. *Biochimica et biophysica acta* **1787**, 1309-1316
256. Huang, S., and Czech, M. P. (2007) The GLUT4 glucose transporter. *Cell metabolism* **5**, 237-252
257. Simonson, D. C., and DeFronzo, R. A. (1990) Indirect calorimetry: methodological and interpretative problems. *The American journal of physiology* **258**, E399-412
258. Lima, G. A., Anhe, G. F., Giannocco, G., Nunes, M. T., Correa-Giannella, M. L., and Machado, U. F. (2009) Contractile activity per se induces transcriptional activation of SLC2A4 gene in soleus muscle: involvement of MEF2D, HIF-1a, and TRalpha transcriptional factors. *American journal of physiology. Endocrinology and metabolism* **296**, E132-138
259. Zhang, H., Zhang, G., Gonzalez, F. J., Park, S. M., and Cai, D. (2011) Hypoxia-inducible factor directs POMC gene to mediate hypothalamic glucose sensing and energy balance regulation. *PLoS biology* **9**, e1001112
260. Adler, L. t., Chen, C., and Koutalos, Y. (2014) Mitochondria contribute to NADPH generation in mouse rod photoreceptors. *The Journal of biological chemistry* **289**, 1519-1528
261. Rasmussen, H., Zawalich, K. C., and Zawalich, W. S. (1995) Metabolic activation of Ca(2+)-independent phosphoinositide hydrolysis in beta-cells and its role in the control of insulin secretion. *Endocrinology* **136**, 577-584
262. Aizawa, T., Komatsu, M., Sato, Y., Ishihara, F., Suzuki, N., Nishii, N., Hashizume, K., and Yamada, T. (1994) Insulin secretion by the pancreatic beta cell of aged rats. *Pancreas* **9**, 454-459
263. Fransson, U., Rosengren, A. H., Schuit, F. C., Renstrom, E., and Mulder, H. (2006) Anaplerosis via pyruvate carboxylase is required for the fuel-induced rise in the ATP:ADP ratio in rat pancreatic islets. *Diabetologia* **49**, 1578-1586

264. Sugden, M. C., and Holness, M. J. (2011) The pyruvate carboxylase-pyruvate dehydrogenase axis in islet pyruvate metabolism: Going round in circles? *Islets* **3**, 302-319
265. Willenborg, M., Panten, U., and Rustenbeck, I. (2009) Triggering and amplification of insulin secretion by dimethyl alpha-ketoglutarate, a membrane permeable alpha-ketoglutarate analogue. *European journal of pharmacology* **607**, 41-46
266. MacDonald, M. J. (1982) Evidence for the malate aspartate shuttle in pancreatic islets. *Archives of biochemistry and biophysics* **213**, 643-649
267. Fallon, M. J., and MacDonald, M. J. (2008) Beta-cell alpha-ketoglutarate hydroxylases may acutely participate in insulin secretion. *Metabolism: clinical and experimental* **57**, 1148-1154
268. Henquin, J. C. (2000) Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* **49**, 1751-1760
269. Proks, P., Reimann, F., Green, N., Gribble, F., and Ashcroft, F. (2002) Sulfonylurea stimulation of insulin secretion. *Diabetes* **51 Suppl 3**, S368-376
270. Navarro-Tableros, V., Fiordelisio, T., Hernandez-Cruz, A., and Hiriart, M. (2007) Physiological development of insulin secretion, calcium channels, and GLUT2 expression of pancreatic rat beta-cells. *American journal of physiology. Endocrinology and metabolism* **292**, E1018-1029
271. Jing, X., Li, D. Q., Olofsson, C. S., Salehi, A., Surve, V. V., Caballero, J., Ivarsson, R., Lundquist, I., Pereverzev, A., Schneider, T., Rorsman, P., and Renstrom, E. (2005) CaV2.3 calcium channels control second-phase insulin release. *The Journal of clinical investigation* **115**, 146-154
272. Corpeleijn, E., Saris, W. H., and Blaak, E. E. (2009) Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle. *Obesity reviews : an official journal of the International Association for the Study of Obesity* **10**, 178-193
273. Kelley, D. E., Reilly, J. P., Veneman, T., and Mandarino, L. J. (1990) Effects of insulin on skeletal muscle glucose storage, oxidation, and glycolysis in humans. *The American journal of physiology* **258**, E923-929
274. Koska, J., Ortega, E., Bogardus, C., Krakoff, J., and Bunt, J. C. (2007) The effect of insulin on net lipid oxidation predicts worsening of insulin resistance and development of type 2 diabetes mellitus. *American journal of physiology. Endocrinology and metabolism* **293**, E264-269
275. Cerf, M. E. (2006) Transcription factors regulating beta-cell function. *European journal of endocrinology / European Federation of Endocrine Societies* **155**, 671-679
276. Kaneto, H., Nakatani, Y., Miyatsuka, T., Matsuoka, T. A., Matsuhisa, M., Hori, M., and Yamasaki, Y. (2005) PDX-1/VP16 fusion protein, together with NeuroD or Ngn3, markedly induces insulin gene transcription and ameliorates glucose tolerance. *Diabetes* **54**, 1009-1022
277. DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J., and Felber, J. P. (1981) The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* **30**, 1000-1007
278. Shulman, G. I., Rothman, D. L., Jue, T., Stein, P., DeFronzo, R. A., and Shulman, R. G. (1990) Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by 13C nuclear magnetic resonance spectroscopy. *The New England journal of medicine* **322**, 223-228
279. Cline, G. W., Petersen, K. F., Krssak, M., Shen, J., Hundal, R. S., Trajanoski, Z., Inzucchi, S., Dresner, A., Rothman, D. L., and Shulman, G. I. (1999) Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes. *The New England journal of medicine* **341**, 240-246

280. Richter, E. A., and Hargreaves, M. (2013) Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiological reviews* **93**, 993-1017