First phase insulin secretion

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Declaration

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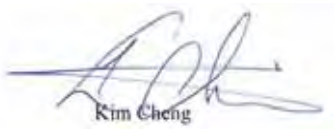
Signed 

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“Never, never, never give up”

- *Winston Churchill*

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That's all I can think of right now. I hope I haven't left anyone important out but if I have, I sincerely apologise and my thanks to you too.

Abbreviations

1,3-BPG	1,3-bisphosphoglycerate
2PG	2-phosphoglycerate
3PG	3-phosphoglycerate
7AAD	7-amino-actinomycin D
α-KGDHC	α-ketoglutarate dehydrogenase complex
ADP	Adenosine diphosphate
AHR	Aryl hydrocarbon receptor
Akt2	RAC-β serine/threonine protein kinase
Aldo	Aldolase
Arnt	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
AUC	Area under the curve
BCS	Bovine calf serum
bHLH-PAS	Basic helix-loop-helix Per-ARNT-single minded
BMI	Body mass index
BrdU	5-bromo-2-deoxyuridine
CHREBP	Carbohydrate-responsive element-binding protein
CIIIT	Chronic intermittent intravenous insulin therapy
COX	Cytochrome *c* oxidase
CVD	Cardiovascular disease
DAPI	4',6-diamidino-2-phenylindole
DFO	Deferoxamine
DFS	Deferasirox
DMEM	Dulbecco's modified eagle medium
ESRD	End stage renal disease
ETC	Electron transport chain
FACS	Fluorescent automated cell sorting
$FADH_2$	Flavin adenine dinucleotide
FBP	Fructose-1,6-bisphophate
Fbpase	Fructose-1,6-bisphosphatase
FCCP	Cyanide p-trifluoromethoxyphenylhydrazone
FH	Fumurate hydrotase
FIH	Factor inhibiting HIF
FMN	Flavin mononucleotide
GAP	Glyceraldehyde 3-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphotase
G6pi	Glucose-6-phosphoisomerase
GIR	Glucose infusion rate
Gck	Glucokinase
Glut	Glucose transporter
GSIS	Glucose stimulated insulin secretion
GTP	Guanosine triphosphate
GTT	Glucose tolerance test
GWAS	Genome wide association studies
$HbA1_C$	Glycated haemoglobin
HFD	High fat diet
HGP	Hepatic glucose production
Hif-1α	Hypoxia inducible factor-1α
Hk	Hexokinase

IGT	Impaired glucose tolerance
Ir	Insulin receptor
IVGTT	Intravenous glucose tolerance test
K_{ATP}	ATP-sensitive potassium channels
KCl	Potassium chloride
Kir	Potassium inward rectifier
K_m	Michaelis constant
Ldh	Lactate dehydrogenase
MDH	Malate dehydrogenase
MMTT	Mixed meal tolerance test
NAD	Nicotinamide adenine dinucleotide
NEFA	Non esterified fatty acid
Nnt	Nicotinamide nucleotide transhydrogenase
Nampt	Nicotinamide phospho-ribosyl-transferase
PBS	Phosphate buffered solution
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PDP	Pyruvate dehydrogenase phosphatase
Pdx1	Pancreatic duodenal homeobox 1
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PGK	Phosphoglycerate kinase
Pfk	Phosphofructokinase
PK	Pyruvate kinase
PIVIT	Pulsatile intravenous insulin therapy
QH_2	Ubiquinol
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RPMI	Park Memorial Institute-1640 medium
SDH	Succinate dehydrogenase
siRNA	Short interfering RNA
Srebp1c	Sterol regulatory element binding protein 1C
Sur	Sulphonylurea receptor
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCF7L2	Transcription factor 7-like 2
TCA	Tricarboxylic acid
Tfam	Mitochondrial transcription factor A
Ucp	Uncoupling protein
VBC	pVHL-elonginB-elonginC
VDCC	Voltage dependent calcium channel
VEGF	Vascular endothelial growth factor
Vhl	von Hippel-Lindau

Abstract

Type 2 diabetes (T2D) is a metabolic disorder characterised by the inability of β-cells to secrete enough insulin to maintain glucose homeostasis. Pancreatic β-cells secrete insulin in a biphasic manner, first and second phase insulin secretion, and loss of first phase insulin secretion is an independent predictor of T2D onset. Restoration of first phase insulin secretion has been shown to improve blood glucose in T2D by suppressing hepatic glucose production and priming insulin sensitive tissue to more readily take up glucose and has thus prompted numerous studies into its regulation. First phase insulin secretion is initiated primarily by the classical triggering pathway, a complex system comprised of multiple stimulatory signals. The aim of this study was to identify models of reduced first phase insulin secretion. Two models were identified – high passage MIN6 cells and β-cells that have reduced or lack the transcription factor hypoxia inducible factor-1α (HIF-1α).

High passage MIN6 cells were shown to have reduced first phase insulin secretion while still retaining some second phase secretory capacity, similar to that seen in T2D patients early on in disease progression. When compared to functioning low passage MIN6 cells, high passage cells displayed an altered metabolic profile including significantly reduced intracellular ATP content, reduced glucose and lipid oxidation, reduced glucose uptake, and altered gene expression profile. The changes observed are also similar to that seen in the β-cells of T2D patients.

Mice lacking HIF-1α in the β-cells have reduced glucose tolerance and glucose stimulated first phase insulin secretion. These β-cells also have reduced intracellular ATP content. MIN6 cells with reduced HIF-1α display similar characteristics of reduced insulin first phase insulin and intracellular ATP content. Increasing HIF-1α with the iron chelator Deferasirox in mice on a high fat diet improved their glucose

tolerance. The results of this study showed that HIF-1α is essential for normal β-cell function, contrary to previously published reports.

Publications, presentations, and prizes arising from this thesis

Publications

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Chapter 1

Introduction

1.1 The pancreas and the islets of Langerhans

The pancreas is an organ of predominantly exocrine, endocrine, and ductal cells. The functions of cell types in the pancreas vary, with the acinar cells providing for the exocrine functions, the ducts for exocrine secretion, and islets of Langerhans, or simply referred to as islets, performing the endocrine functions. The islets were first discovered in 1869 and have four major endocrine cell types: α-cells, β-cells, δ-cells, and γ-cells, with the β-cells making up between 66 – 74 % of the islet mass [1]. Islets as a whole make up only a small percentage of the pancreatic volume (1-2%). The β-cells secrete the hormone insulin which is required for glucose homeostasis.

1.2 Diabetes

Diabetes mellitus is a disease which manifests as the inability to control blood glucose levels. It is now considered a worldwide epidemic. It was estimated that in 2010, 220 million people worldwide had diabetes, with this figure predicted to rise to 366 million people by 2030 [2]. Diabetes is classified into three major groups: Type 1, Type 2, and gestational diabetes. Even though all groups have the same disease outcome (high blood glucose levels), they are different in how disease onset occurs.

Type 1 diabetes (T1D) is an autoimmune disease whereby the immune system attacks and destroys the pancreatic β-cells responsible for producing insulin. This results in a near complete lack of insulin and thus the patient is unable to maintain homeostasis in blood glucose [3]. Gestational diabetes is defined as glucose intolerance first diagnosed in pregnancy. Type 2 diabetes (T2D) is a metabolic disorder and is characterised by the inability of β-cells to produce enough insulin to maintain glucose homeostasis. Most patients also have insulin resistance in peripheral tissues. To compensate for insulin resistance in peripheral tissues, normal β-cells produce more insulin. If β-cell function deteriorate over time due to this compensation, then

maintenance of blood glucose levels may no longer be possible and glucose intolerance ensues [4].

1.3 Factors contributing to T2D

T2D is a complicated disease, the development of which can be caused by a number of factors, both environmental and genetic.

1.3.1 Environmental factors

1.3.1.1 Obesity

The environment and personal lifestyle are major factors in T2D development. It has long been established that obesity and increased body fat have a clear role in the worsening of glucose tolerance and reduced insulin sensitivity [5-7]. According to the World Health Organisation, a person is overweight if they have a body mass index (BMI = weight (kg)/height $(m)^2$) greater than or equal to 25 and obese if their BMI is greater than or equal to 30. Regardless of sex, people with a BMI of greater than 25 have an increased risk of T2D development [5-7]. In addition to this, more children and adolescents are becoming overweight or obese and T2D is emerging as a critical health issue in this population. One study in the United States has claimed a 10-fold increase in incidence of T2D in children and adolescents between 1982 and 1994 [8]. A study by Sinha and colleagues [9] showed that 25 % of 55 obese children and 21 % of 112 obese adolescents had impaired glucose tolerance (IGT). The reasons for increasing risk of T2D in children and adolescents are varied and are probably attributable to diet and sedentary lifestyle as discussed later.

Obesity and insulin resistance are inter-related because adipose tissues are not simply storage units for fat but play an active role in metabolism. Adipose tissues secrete a number of factors including non-esterified fatty acids (NEFA), glycerol,

hormones, and proinflammatory cytokines. Obese and people with T2D have increased NEFA levels [10, 11] and it has been postulated that the release of NEFAs may be an important factor linking increased adipose tissue and insulin resistance [12]. An increase in NEFAs may compete with glucose for substrate oxidation and can also lead to a cascade of events resulting in reduced insulin receptor signalling [12]. NEFA also has an effect β-cell function and survival [13]. It has been shown that NEFAs may induce endoplasmic reticulum stress in β-cells early in T2D disease progression and high glucose amplifies this effect in clonal β-cells [14]. NEFAs have also been shown to induce a mild inflammatory response *in vitro*, similar to that seen in T2D islets [15].

1.3.1.2 Diet and exercise

Obesity can be broadly defined as the result of a long term excess of energy intake compared to energy expenditure, leading to storage of excess energy as adipose tissue [16]. A number of studies have confirmed that increased dietary fat is associated with impaired glucose tolerance and insulin resistance in both humans and animals [17-21]. Unfortunately, the increase in incidences of T2D can be correlated to the increase in dietary fat being consumed over time, the average fat intake in an American diet is now greater than 30 %, with the top 20 % of the population consuming more than 46 % of their calorie intake from fat [22, 23].

An increase in fat consumption in addition to a sedentary lifestyle only compounds the problem of obesity. Lack of physical activity and an increased sedentary behaviour has been shown to be associated with the metabolic syndrome and T2D [24, 25]. Patients already suffering from T2D benefit from exercise regimes. It has been shown that exercise, in the form of three workouts a week (average of 53 minutes per workout with a combination of cardio and resistance training), reduced glycated haemoglobin (HbA_{1C}) by 0.66 % compared to non-exercise control groups

[26]. This magnitude of change is comparable to the 0.7 % change seen in the United Kingdom Prospective Diabetes Study (UKPDS) between conventional and intensive glucose lowering therapy [27].

1.3.2 Genetic factors

Even though environmental factors play a large role in T2D onset, genetic factors also have an important role. Approximately 5-10 % of apparent T2D cases are due to monogenic mutations. Genes that have been shown to regulate insulin secretion will be discussed in greater detail in Section 1.9 [28]. There is substantial evidence from studies both in families [29] and work with monozygotic twins [30] for a strong genetic component of T2D. Correspondingly, recent studies using large scale, genome wide association studies (GWAS) have identified ~40 susceptibility loci. Polymorphisms within these *loci* increase the risk of T2D and, in the majority of cases, this is through changes in β-cell function [31-34]. The identification of such susceptibility *loci* has prompted a number of subsequent studies which have sought to explore the molecular mechanisms through which the implicated genes may impact on β-cell function or survival using rodent models and cellular systems [35-38]. Polymorphisms in the transcription factor 7-like 2 (*TCF7L2*) and calpain-10 are associated with reduced first phase insulin secretion [39] and increased risk of T2D in Mexican Indians respectively [28].

1.4 Complications of T2D

Besides the obvious outcome that T2D patients are unable to control blood glucose, patients are at risk of other disease complications. Among the major complications associated with T2D are cardiovascular diseases and microvascular complications leading to blindness and renal failure. The link between glycaemic control and

cardiovascular disease (CVD) has long been suspected, with the first associations made in 1965 by groups in the UK and the US [40, 41]. It wasn't until Uusitupa and colleagues in 1993 [42] that established a clear link between T2D and CVD. It was previously shown that patients with T2D are two to six times more likely to die from CVD than people without diabetes [43] and CVD accounts for up to 80 % of deaths in T2D patients [44]. However, a recent meta analysis has shown that intensive glycaemic control did not alter the risk of cardiovascular mortality [45] and thus this issue of causality will require further examination.

Another major complication of T2D is end stage renal disease (ERSD). Diabetic nephropathy is the major cause of ERSD and is due to the damage in the kidneys caused by persistently high blood glucose. Approximately 25 % - 40 % of T2D patients will have diabetic nephropathy 25 years after disease onset [46, 47]. At present, the best methods to reduce the chances of diabetic nephropathy are by careful control of blood glucose or by anti-hypertension drugs to reduce blood pressure [48].

1.5 Insulin secretion

Pancreatic β-cells secrete insulin in a biphasic manner, defined as first and second phase (Figure 1.1). It is thought that the initial insulin spike is crucial for glucose homeostasis. Studies have shown that loss of first phase insulin secretion is an independent predictor of T2D [49-53]. Type 1 diabetic patients also lose first phase insulin secretion during the prediabetic period [54]. Disease states associated with impairment in first phase insulin secretion such as Huntington's and Alzheimer's diseases are also associated with increased risk of diabetes [55, 56]. Before the development of frank diabetes, people with fasting hyperglycaemia lack first phase insulin secretion [57] and people with impaired glucose tolerance (IGT) have reduced plasma insulin levels after a glucose load [58-60]. This section will focus on first phase

insulin secretion in T2D and recent advances on how first phase insulin secretion is regulated.

First phase insulin secretion by definition occurs rapidly, with peak values usually achieved 1-2 minutes after glucose stimulation [61]. Quite differently, second phase insulin secretion is more gradual and long lasting, usually reaching a plateau 25-30 minutes after stimulation in people with normal glucose tolerance [61] (Figure 1.1). While both phases of insulin secretion play an important role in glucose homeostasis, the relative importance of first phase insulin secretion may be greater. Studies in humans where first phase but not second phase insulin secretion have been artificially blocked by an infusion of somatostatin had significantly worse glucose tolerance compared with control conditions [62]. First phase insulin secretion has a strong effect to suppress hepatic glucose production (HGP) and patients with T2D have an inappropriate increase in HGP [63]. Insufficient suppression of HGP leads to IGT [64-66]. First phase insulin secretion also primes other insulin sensitive tissues, including muscle, to more rapidly take up glucose [67].

In addition to the biphasic manner of insulin secretion, effective control of glucose homeostasis requires secretion of insulin to be oscillatory [68]. These oscillations occur every 9–14 minutes in humans *in vivo* [69] while others have reported shorter time periods (5–8 minutes) in isolated rat islets [70]. The oscillatory nature of insulin secretion is due to oscillations in glycolysis and its regulatory enzymes [71, 72]. Subjects with T2D have abnormal oscillations in insulin secretion [73] and together with the fact that they also have reduced glucose oxidation [74] could contribute to inhibited first phase insulin secretion. Some first degree relatives of people with T2D also have abnormal oscillations [75] and thus would suggest some genetic and / or environmental regulation. Insulin infused with an oscillatory pattern has a greater effect than continuous delivery for individuals with diabetes [68] and as such, the less

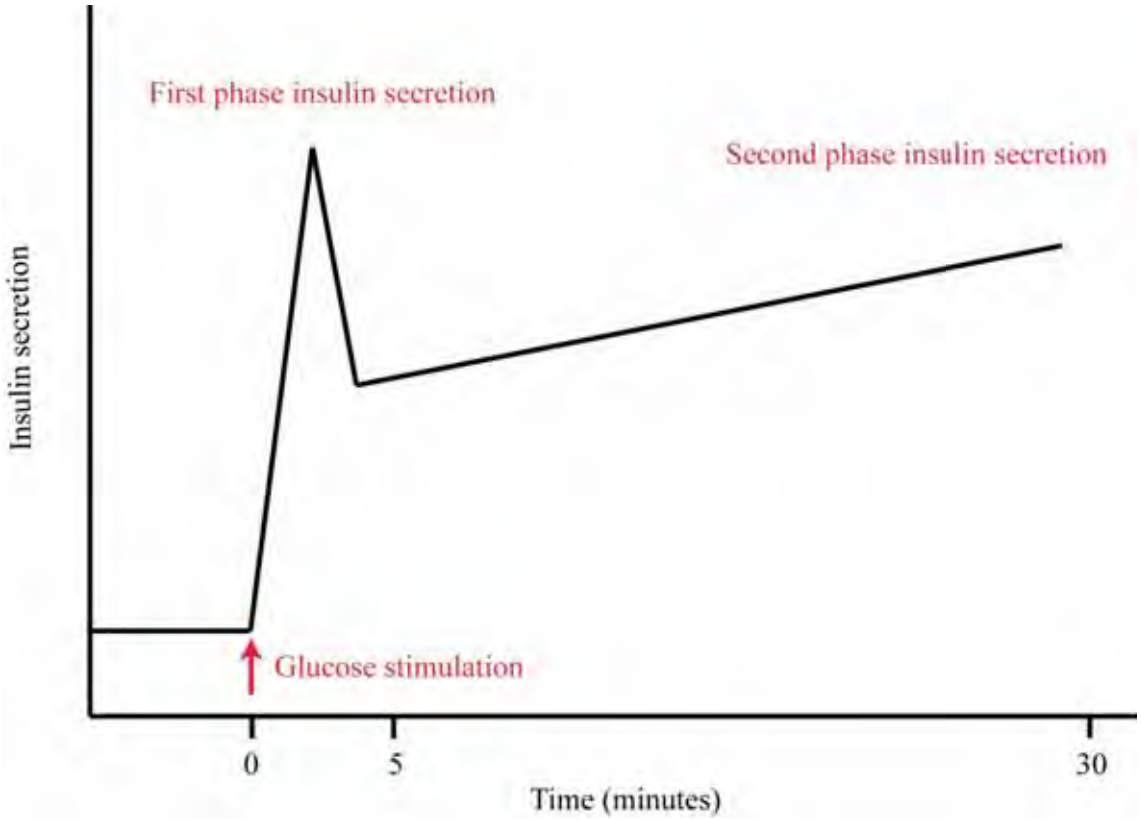

Figure 1.1 First and second phase insulin secretion in the β-cell. First phase insulin secretion occurs very rapidly, usually within 10 minutes of glucose stimulation. Second phase insulin secretion is more gradual and long lasting, occurring 25 – 30 minutes after glucose stimulation [61].

common pulsatile intravenous insulin therapy (PIVIT) or chronic intermittent intravenous insulin infusion therapy (CIIIT) has been used to greater effect [76, 77]. The exact mechanism(s) as to why it is more effective remains to be elucidated but a proposed theory is that PIVIT allows greater expression of insulin receptors than continuously high insulin levels thus increasing sensitivity in peripheral tissues [77].

1.5.1 Measurement of first phase insulin secretion

In order to measure biphasic insulin secretion *in vivo*, the following three methods are commonly employed: a) the intravenous glucose tolerance test (IVGTT), b) the hyperglycaemic clamp [78, 79], and c) mixed meal tolerance tests (MMTT) [80, 81]. The IVGTT is relatively simpler to conduct compared with the hyperglycaemic clamp. It involves a bolus of glucose injected intravenously over a short period (typically 1 minute) and blood samples taken at many, specific time points to measure insulin secretion and/or C-peptide [78]. This method is well suited for testing first phase insulin secretion as after the initial glucose injection, no further glucose is introduced into the system. There are issues with variability in readings of the initial insulin spike as glucose decays differently in each subject depending on their glucose tolerance [82]. In normal individuals, approximately 50 % of secreted insulin is cleared by the liver in the first pass of metabolism. So, if C-peptide is measured, deconvolution analysis can be used to estimate actual insulin secretion.

The hyperglycaemic clamp was first described in 1979 by Ralph DeFronzo and colleagues [83] and is a sophisticated technique which requires time and experienced labour. During this procedure, the subject's glucose level is raised to a pre-determined level, for example 125 mg/dl (7 mM/L) above basal levels by a priming dose of intravenous glucose. The hyperglycaemic plateau is then maintained by a constant infusion of glucose. Insulin concentrations, C-peptide concentrations and the glucose

infusion rate (GIR) are used as measures of insulin secretion and action [83]. As the β-cells are being exposed to glucose for a longer period of time, it is a more accurate measurement of both first and second phase insulin secretion and is considered the gold standard for the measurement of insulin secretion *in vivo* [82].

The MMTT, also known as the Boost test, is a relatively simple test and is suitable when performing a hyperglycaemic clamp is not feasible, such as in epidemiologic studies or for following individual changes in insulin secretion [80]. In a MMTT, subjects fast overnight then ingest a liquid meal (e.g. Sustacal™/Boost™) with a known proportion of carbohydrates, protein, and fat. Common values are 55 % carbohydrate, 25 % protein, and 20 % fat. Blood samples are taken at specific time points to analyse glucose, insulin, and C-peptide levels [80, 81]. Although not considered the gold standard for the determination of insulin secretion, it represents a more physiological response as the stimulus is delivered orally, and induces the normal incretin response [81]. A recent study showed that MMTT provided comparable results at 15 minutes after intake to results from a hyperglycaemic clamp in children with normal glucose tolerance [80]. The MMTT is clearly physiologically relevant as the normal means of glucose exposure is oral, not intravenous.

1.5.2 Insulin granule pools

The insulin that is secreted to maintain glucose homeostasis exists as preformed insulin granules within the β-cells. Secretory granules reside in two main pools; the docked and reserve pools [84]. The docked pool can be further divided into granules that are immediately releasable and readily releasable, and whether they are primed or un-primed [85]. Estimates suggest approximately 13000 granules in the docked pool and 50 granules per β-cell in the immediately releasable pool in normal mice [85, 86]. The 50 granules docked and immediately releasable are located in close proximity to the

calcium channels and was previously thought to be utilised for first phase insulin secretion [86]. The second phase of insulin secretion is thought to be maintained by a subset of approximately 1000 granules which are docked but un-primed [85]. In order for the granules to be primed and readily releasable, they must go through a series of reactions that involve adenosine triphosphate (ATP), calcium, and temperature [87, 88].

However, more recent experiments have described a new model of insulin granule secretion mediated by cAMP and Epac2/Rap1 signalling [89-91]. This new model is based on three distinct modes of insulin granule dynamics: "Old face" where predocked granules are fused to the membrane by stimulation, "Restless newcomer" where insulin granules are immediately recruited and fused to the membrane by stimulation, and "Resting newcomer" where insulin granules are recruited, docked, and are then fused to the membrane by stimulation [89]. In this new model of insulin secretion, both first and second phase insulin secretion is mediated by the "Restless newcomer" insulin granules. However, the two phases of insulin secretion are produced from different pools of these "Restless newcomers" and the mechanisms behind it are distinct. Possible mechanisms differentiated first and second phase insulin secretion may involve actin remodelling, cAMP, and Epac2/Rap1 signalling [90, 91].

1.6 Energy homeostasis

In order for a cell to carry out its numerous functions within an organism, it needs a constant supply of energy. Most organisms, whether plant or animal, utilise ATP as a source of energy. In relation to glucose homeostasis, ATP is required for the secretion of insulin as discussed previously. Phototrophs (plants and certain bacteria) synthesise ATP through a process involving sunlight known as photosynthesis. Chemotrophs obtain their source of energy by oxidising organic compounds such as carbohydrates, lipids, and proteins. The oxidation of these organic compounds for the synthesis of

ATP occurs via the actions of glycolysis, the TCA cycle, and oxidative phosphorylation [92].

1.6.1 Glycolysis

Glycolysis occurs in the cell cytosol and is the process by which glucose is converted to pyruvate, yielding two molecules of ATP per molecule of glucose in the process. The glycolytic pathway is nearly ubiquitous in all living organisms. It is an important process not only to provide a small amount of ATP but to provide precursor components for the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, which will be discussed later. Glycolysis, as shown in Figure 1.2, consists of ten enzymatic reactions and is functional in both aerobic and anaerobic states [93].

The first step of the glycolytic pathway is the phosphorylation of glucose to glucose-6-phosphate (G6P) by glucokinase (GCK, hexokinase IV). GCK is seen as the rate limiting step in glycolysis with a very narrow tissue distribution, as protein and mRNA expression has only been observed in the liver and pancreatic islets and not in the brain, spleen, intestinal mucosa, pancreas, kidney or white adipose tissue [94, 95]. Due to the high affinity of GCK to glucose compared to other hexokinases in its family, and the fact that mutations in the GCK gene leads to impaired insulin secretion and diabetes [96-98], GCK has been proposed to be the glucose sensor in an organism [99].

The next two reactions in glycolysis utilise ATP to function; phosphoglucose isomerise (PGI) converts G6P to fructose-6-phosphate (F6P) and then phosphofructokinase (PFK) phosphorylates F6P in an irreversible action to produce fructose-1,6-bisphophate (FBP). Substrates for PGI are not only utilised in glycolysis but also for gluconeogenesis, the pentose phosphate cycle, glycoprotein synthesis, and inositol synthesis [93]. Interestingly, PGI behaves as a cytokine extracellularly and

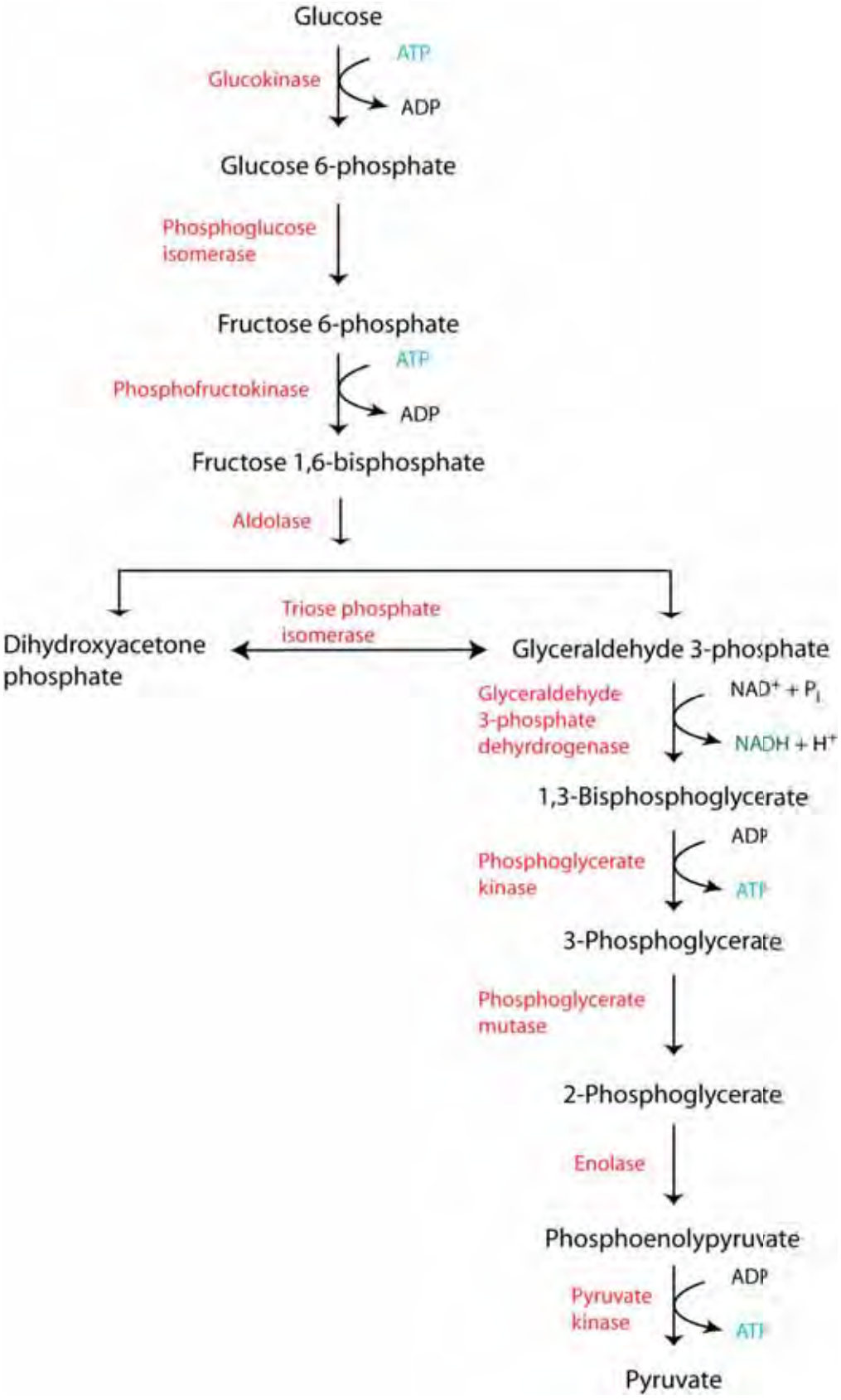

Figure 1.2 The glycolytic pathway. The glycolytic pathway consists of ten enzymatic reactions to metabolise glucose to pyruvate. For every one molecule of glucose metabolised, one molecule of NADH and two molecules of ATP are produced. Figure modified from [93].

experiments have identified PGI as an autocrine motility factor, a cytokine secreted by tumours to stimulate cell migration and metastasis [100].

Three types of PFK cDNA have been identified and mapped to different chromosomes in humans; muscle (PFK-M), liver (PFK-L), and platelet type (PFK-P) [101]. Both PGI and the PFK isoforms are regulated by hypoxia, specifically via pathways involving the hypoxia responsive complex hypoxia inducible factor 1 (HIF-1) [102, 103].

After the uni-directional action of PFK, FBP is subsequently converted to glyceraldehyde 3-phosphate (GAP) by the enzyme aldolase. In addition to this, the actions of aldolase also produce dihydroxyacetone phosphate, an intermediate that can be readily converted to GAP by triose phosphate isomerase [93]. There are three types of aldolase enzymes that have been identified; aldolase A (found in the muscle), aldolase B (expressed predominantly in the liver), and aldolase C (expressed predominantly in the brain) [92].

The reactions described so far have yet to create any ATP; in fact, two of the enzymes have utilised ATP in order to perform their function. The first reaction in glycolysis to generate a high energy intermediate is the conversion of GAP to 1,3-bisphosphoglycerate (1,3-BPG) by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [93]. The role of GAPDH has traditionally been viewed as simply glycolytic, however emerging evidence seems to suggest more important functions in other areas, although these are less well understood. Nuclear expression of GAPDH protein has been shown to participate in DNA repair and regulating transcription, while other groups have implicated GAPDH as an active participant in neurodegenerative conditions such as Huntington's and Parkinson's disease [104, 105].

With the formation of 1,3-BPG in the previous step, the first molecule of ATP to be generated in the glycolytic pathway is set to occur. In this reaction,

phosphoglycerate kinase (PGK-1) transfers the phosphoryl group from 1,3-BPG to adenosine diphosphate (ADP), yielding 3-phosphoglycerate (3PG) and one molecule of ATP as its products [93]. Another isoform of phosphoglycerate kinase exists (PGK-2), however this isoform is very tissue specific (testes and sperm) and is only expressed during the late stages of spermatogenesis [106]. Similar to GAPDH, PGK-1 has been shown to have a wide range of functions outside of the glycolytic pathway, ranging from DNA replication and repair [107] to inhibiting tumour cell progression [108]. The *PGK-1* gene also contains hypoxia response elements and is regulated by HIF-1 in a manner similar to PGI and PFK as described previously [109].

The next series of reactions lead to the generation of the second molecule of ATP via glycolysis. Firstly, phosphoglycerate mutase (PGM) catalyses the conversion of 3PG to 2-phosphoglycerate (2PG). 2-phosphoglycerate is then used to form anther high energy intermediate, phosphoenolpyruvate (PEP), by the enzyme enolase [93]. Again, similar to other genes in the glycolytic pathway, enolase is regulated in an oxygen dependent manner by HIF-1 [110]. In the last glycolytic reaction, PEP is converted to pyruvate and one molecule of ATP by the enzyme pyruvate kinase (PK) [93]. In mammals, PK exists as four isoenzymes (L-, R-, M_1-, M_2- types), each with their own tissue specificity. In the liver, kidney, small intestine, and pancreatic β-cells is the isoenzyme L-PK while R-PK is only expressed in erythrocytes. The M_1-PK isoenzyme is found in the skeletal muscle, heart, and brain while M_2-PK is a ubiquitous form, found in nearly all cell types [111]. From here, pyruvate can be used as a substrate for the citric acid cycle if enough oxygen is available or otherwise be recycled to enable the continued operation of the glycolytic pathway in anaerobic conditions.

1.6.1.1 Anaerobic glycolysis

In certain circumstances, such as in muscle during vigorous exercise, oxygen levels may be depleted and it is necessary for the glycolytic pathway to keep functioning to provide the cell with ATP. In this case, pyruvate is not shuttled off but instead recycled and converted to lactate and nicotinamide adenine dinucleotide (NAD^+) through the actions of lactate dehydrogenase (LDH). The substrate NAD^+ is necessary for the GAPDH to function, which cells have in limited supply. As such, the glycolytic pathway is able to continue breaking down glucose to produce ATP under anaerobic conditions [92].

The enzyme LDH functions as a tetramer, made from a combination of the different subunits – LDH-A found predominantly in muscle tissue, LDH-B found predominantly in the heart, and LDH-C found only in the testes and spermatozoa. It has been recently shown that another LDH gene, LDH-A like 6A (*LDHL6A*), exists exclusively in human testes and plays a role in spermatogenesis [112].

1.6.2 Gluconeogenesis

In times of starvation where diet does not supply sufficient glucose for cellular function, pyruvate can be converted to glucose through the gluconeogenesis pathway (Figure 1.3). This is a particularly important metabolic pathway as the brain requires glucose as its major source of fuel [93]. The major site for gluconeogenesis is the liver. The kidney cortex also contains the necessary machinery, however the amount of glucose

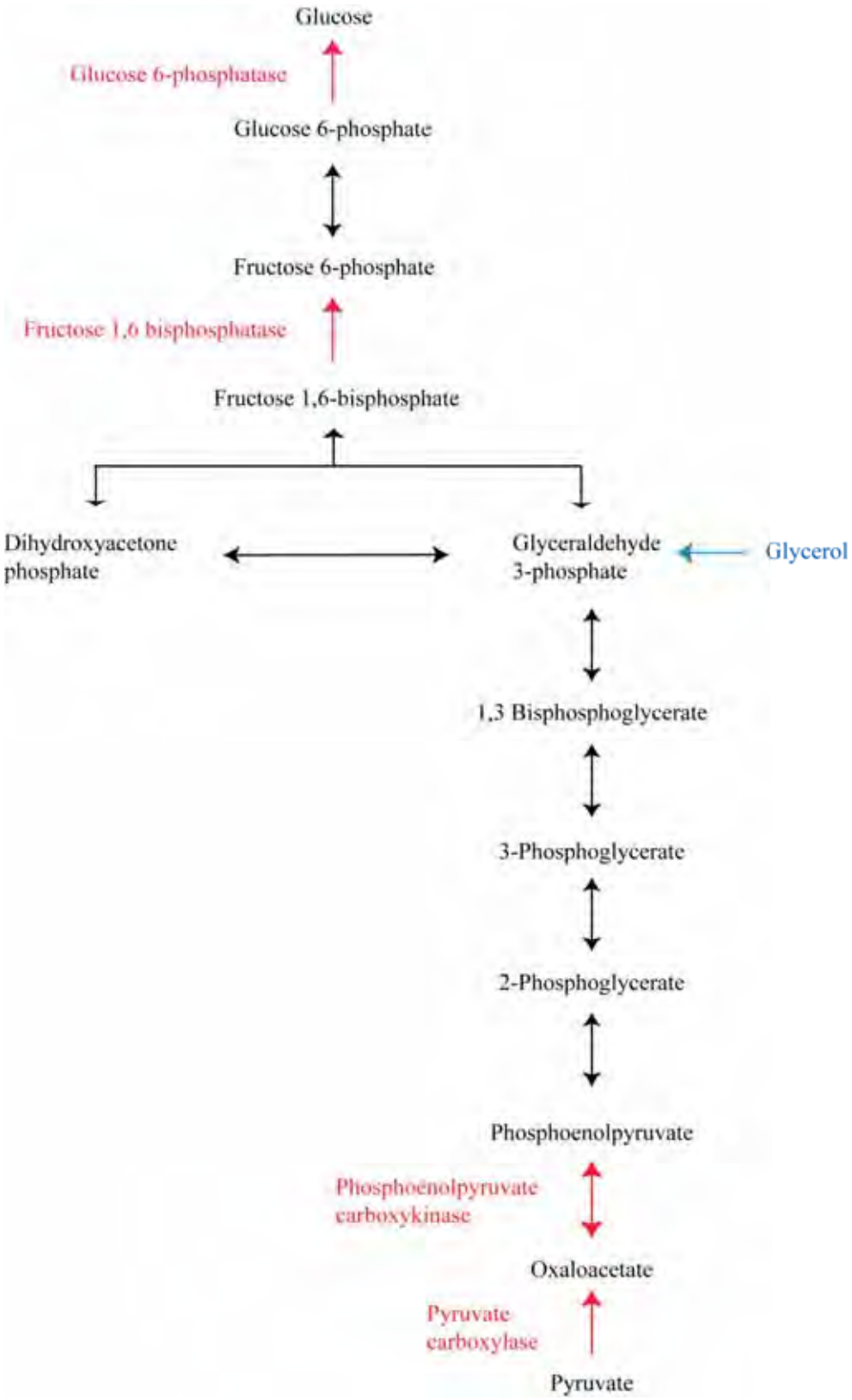

Figure 1.3 The gluconeogenic pathway. The gluconeogenic pathway converts pyruvate to glucose and makes up part of endogenous glucose production. Many of the reactions of gluconeogenesis are common to glycolysis, with the distinctive gluconeogenic reactions shown in red. The entry point for glycerol is shown. Figure modified from [93].

produced in the kidney cortex is smaller, probably due to less mass [113]. Many of the enzymes are shared between glycolysis and gluconeogenesis as they are bi-directional. However, there are several key regulatory and uni-directional enzymes that are specific to gluconeogenesis.

Gluconeogenesis begins with the conversion of pyruvate to PEP via a two step process. First, one molecule of ATP is utilised to convert pyruvate into oxaloacetate by pyruvate carboxylase, and subsequent conversion of oxaloacetate to PEP [114] by PEP carboxykinase (PEPCK) [93]. The enzyme PEPCK has been extensively studied. Overexpressing PEPCK in the liver or kidney of animal models leads to peripheral insulin resistance, a characteristic of T2D [115, 116].

Gluconeogenesis continues on by using the same enzymes as in glycolysis until the formation of FBP. As the phosphorylation of F6P by PFK is irreversible, gluconeogenesis uses fructose 1,6-bisphosphatase (FBPase) to convert FBP to F6P [93]. Similar to PEPCK, FBPase may also play an important role in the development of T2D, as type 2 diabetic patients have increased gluconeogenesis from glycerol, a substrate that enters into the gluconeogenic pathway immediately prior to FBPase [114]. Increased expression of FBPase has been observed in animal models of obesity and insulin resistance [117, 118] and expression of human FBPase in the liver of mice leads to an increase in glycerol gluconeogenesis [119]. Converting F6P to FBP consumes ATP and if FBP is converted back to F6P, will create a futile, ATP consuming cycle.

Whereas the previous steps of gluconeogenesis take place in the cytosol, the final step sees G6P transported to the lumen of the endoplasmic reticulum to be converted to glucose by glucose 6-phosphotase (G6Pase; [93]. Similar to both PEPCK and FBPase, where overexpression of either increases endogenous glucose production resulting in symptoms of T2D, overexpression of G6Pase in the liver of mice causes hyperglycaemia and hyperinsulinaemia [120]. The same study also found an increased

in expression of G6Pase in the Zucker diabetic fatty rat model of obesity and T2D [120].

1.6.3 Tricarboxylic acid cycle

Often referred to as the citric acid cycle or the Krebs cycle, the TCA cycle continues on from glycolysis to produce substrates that are utilised in oxidative phosphorylation to metabolise ATP under aerobic conditions (Figure 1.4). The reactions of the TCA cycle were characterised in the 1930's by Albert Szent-Gyorgyi and Hans Krebs. The TCA cycle is at the centre of the metabolic system, oxidising carbohydrates, fatty acids, and amino acids, and generates a number of precursors for other biochemical systems in the cell. Unlike glycolysis, the reactions of the TCA cycle occur inside the mitochondria of eukaryotes rather than the cytosol and is a process involving eight enzymatic reactions. None of the reactions in the TCA cycle require ATP to function [93].

A necessary substrate is required for the commencement of the TCA cycle and this is produced when pyruvate from glycolysis is converted into acetyl coenzyme A (acetyl CoA) by the pyruvate dehydrogenase complex (PDC). The PDC is a very large multi-enzyme complex made up from three enzymes – pyruvate dehydrogenase (E_1), dihydrolipoyl transacetylase (E_2), and dihydrolipoyl dehydrogenase (E_3) [92]. As the PDC serves as the link between glycolysis and the TCA cycle, it is a key reaction in the maintenance of glucose homeostasis in mammals and is regulated via the actions of specific pyruvate dehydrogenase kinases (PDK) and pyruvate dehydrogenase phosphatases (PDP). The expression of both PDKs and PDPs can be altered by a number of factors, including nutrition and hormonal factors [121], and oxygen levels [122].

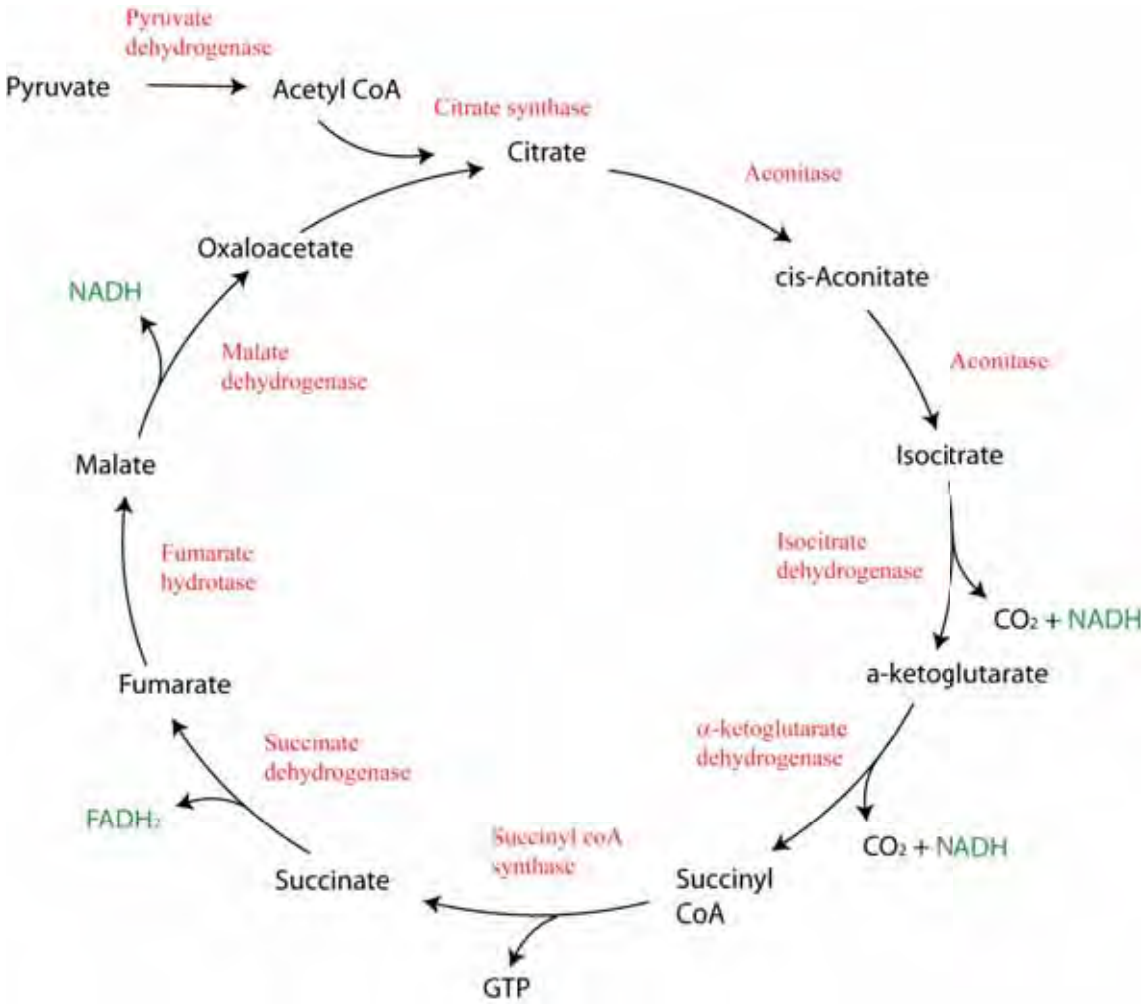

Figure 1.4 The TCA cycle. The TCA cycle occurs in the mitochondria and consists of eight enzymatic reactions to produce substrates for oxidative phosphorylation. Pyruvate, formed from glycolysis, is a necessary substrate for the TCA cycle. For every molecule of pyruvate, three molecules of NADH, one molecule of $FADH_2$, and one molecule of GTP is produced. Enzymes of the TCA cycle are shown in red. Figure modified from [93].

Acetyl CoA is itself a high energy compound and is the primary substrate that enters into the TCA cycle. In the first reaction, citrate synthase catalyses the conversion of acetyl CoA, together with oxaloacetate, to produce citrate [92]. Citrate synthase may also play a role in T2D, with a recent study done showing that basal citrate synthase activity was lower in skeletal muscle cultured from patients with T2D when compared to lean controls. Also, citrate synthase did not seem to respond to insulin stimulation whereas increased activity was observed in tissue from lean controls [123].

Citrate can now be isomerised into isocitrate and this occurs in a two step process. Firstly, aconitase performs a dehydration step to convert isocitrate to cis-aconitate and then the same enzyme hydrates this intermediate to produce isocitrate [93]. In eukaryotes, two isoforms of aconitase have been identified – mitochondrial aconitase, which is the catalytic enzyme in this specific step of the TCA cycle and cytosolic aconitase, which is involved in the glyoxylate cycle [124]. Interestingly, in a recent study, aconitase was shown to have an additional role in the mitochondrion by stabilising mitochondrial DNA [125].

The next enzyme in the TCA cycle is also involved in a two step process. The enzyme isocitrate dehydrogenase converts isocitrate into the intermediate oxalosuccinate, which is then subsequently converted into α-ketoglutarate. This reaction also produces the first molecules of nicotinamide adenine dinucleotide (NADH) and CO_2 in the TCA cycle [92]. Isocitrate dehydrogenase may also play a part in preventing hyperglycaemia induced cell death. Under conditions of high glucose, a greater amount of reactive oxygen species (ROS) is produced and this plays a pivotal role in triggering apoptosis. However, a study has shown *in vitro* that isocitrate dehydrogenase increased the amounts of substrates necessary to suppress ROS damage and thus cell death [126].

The fourth enzyme in the TCA cycle is the α-ketoglutarate dehydrogenase complex (α-KGDHC) and this converts α-ketoglutarate to succinyl CoA, with one molecule of NADH being produced in the process. The α-ketoglutarate dehydrogenase complex is very similar to PDC in that it is a multi-enzyme complex consisting of α-ketoglutarate dehydrogenase (E_1), dihydrolipoyl transsuccinylase (E_2), and dihydrolipoyl dehydrogenase (E_3) [92]. The α-KGDHC is considered as the most important enzyme in the TCA cycle due to its highly regulated nature and it being the primary site of control of the metabolic flux. As such, many studies have been conducted regarding α-KGDHC, with it being implicated as both a target and generator of ROS [127] and its implications in Alzheimer's disease [128].

Succinyl CoA is a high energy compound and its hydrolysis by succinyl CoA synthase yields succinate and one molecule of guanosine triphosphate (GTP). Guanosine triphosphate is utilised in cells during protein synthesis and signal transduction but is also readily convertible to ATP by nucleoside diphosphate kinase [93]. The final stage of the TCA cycle proceeds after the formation of succinate and involves the regeneration of oxaloacetate so that the TCA cycle can continue to function.

During this final stage of the TCA cycle, a number of intermediates are formed before the formation of oxaloacetate. Firstly, succinate is dehydrogenised to form fumarate and one molecule of flavin adenine dinucleotide ($FADH_2$) by succinate dehydrogenase (SDH). Succinate dehydrogenase is made up of four subunits (A, B, C, D) and is not only a key step in the TCA cycle but also in oxidative phosphorylation by donating electrons to complex III and reducing the ubiquinone pool [93]. Due to its dual vital roles, SDH has been extensively studied and researchers have identified mutations in any one of the subunits results in wide variety of diseases including paraganglioma, tumours, and phaeochromocytomas [129].

In the final two steps, fumarate is hydrated by fumarate hydrotase (FH) to form L-malate, which is then subsequently oxidised to form oxaloacetate by malate dehydrogenase (MDH). During the last oxidation step, one molecule of NADH is also formed as a by-product. With the completion of one turn of the TCA cycle, a total of three molecules of NADH and one molecule of $FADH_2$ are produced, and these are utilised in oxidative phosphorylation and the production of ATP [92].

1.6.4 Oxidative phosphorylation

Oxidative phosphorylation occurs within the inner mitochondrial membrane and is the process by which ATP is generated by the transfer of electrons from NADH or $FADH_2$ to oxygen by a series of electron transporters (Figure 1.5). In essence, this is achieved by the generation of an electron-motive force, conversion into a proton-motive force and using this force to drive an ATP synthesising assembly. Oxidative phosphorylation is the main energy producing mechanism in aerobic organisms and produces a total of 30-36 molecules of ATP for every molecule of glucose [93].

Oxidative phosphorylation begins with the electron transport chain (ETC), a series of four protein complexes (Complexes I, II, III, and IV) that pump protons across the inner mitochondrial membrane to generate an proton-motive force [92]. The first of these, Complex I, is also known as NADH-coenzyme Q reductase and is the entry point for NADH derived from glycolysis and the TCA cycle. Complex I is a large 850 kD enzyme and contains one molecule of flavin mononucleotide (FMN) and six to seven iron-sulfur clusters (Fe-S; [92]. Complex I binds to NADH and transfers the two electrons from NADH to FMN to produce $FMNH_2$. Electrons from $FMNH_2$ are then transferred to Fe-S, which in turn are then shuttled to coenzyme Q, also known as ubiquinone. In the final step of Complex I, ubiquinone is reduced to form ubiquinol

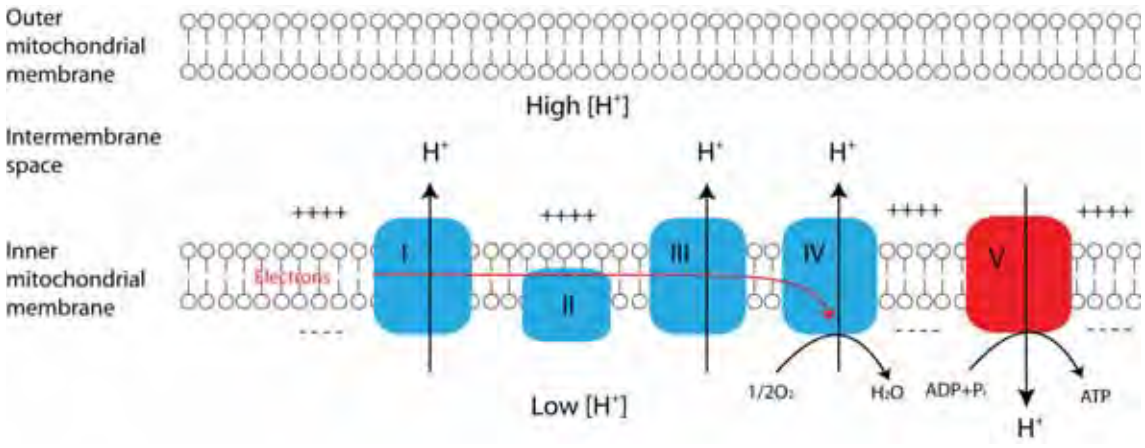

Figure 1.5 Oxidative phosphorylation. The oxidative phosphorylation process occurs in the inner mitochondrial membrane and consists of five protein complexes. NADH and $FADH_2$ from glycolysis and the TCA cycle enter into complex I and II respectively. Complexes I to IV form the ETC and transports electrons across with molecular oxygen as the final electron acceptor. Protons are concomitantly pumped across into the intermembrane space to create a proton-motive force. Complex V, or ATP synthase, utilises the proton-motive force to generate ATP. For every one molecule of glucose, 30-36 molecules of ATP are produced from oxidative phosphorylation. Figure modified from [93].

(QH_2) and the transfer of electrons results in the pumping of four H^+ from the mitochondrial matrix to the intermembrane space [93].

Complex II, also known as succinate-coenzyme Q reductase, of the ETC plays a similar role to Complex I but transfers electrons from $FADH_2$ produced from the TCA cycle rather than NADH. However, unlike the reactions of Complex I, no protons are pumped into the intermembrane space as the catalysed reaction does not produce enough free energy. As such, there is less ATP produced from $FADH_2$ than NADH [93].

The second proton pump in the ETC is Complex III, otherwise known as coenzyme Q-cytochrome *c* reductase. Complex III catalyses the transfer of electrons from QH_2 to cytochrome *c* and it contains two *b*-cytochromes, one cytochrome c_1, and one Fe-S cluster [93]. Electrons from QH_2 are transferred to cytochrome c_1, which in turn are shuttled to cytochrome *c*. Cytochrome *c* is a peripheral membrane protein and binds to both cytochrome c_1 and cytochrome *c* oxidase (COX, Complex IV) and thus acts to transfer electrons between the two complexes. The transfer of electrons in Complex III results in the transport of two H^+ to the intermembrane space [92].

The last set of reactions in the ETC is the transfer of electrons from cytochrome *c* to molecular oxygen, catalysed by Complex IV. Complex IV is a large 200 kD multimeric enzyme consisting of 11-13 subunits in eukaryotes, of which three of these, subunits I, II, and III, are encoded by mitochondrial DNA. In this last reaction, four electrons are transferred to molecular oxygen to completely reduce it to water, while simultaneously pumping protons into the intermembrane space [93]. Complex IV is seen as the rate limiting step in mitochondrial respiration due to its highly regulated nature [130]. In a recent study, Fukuda et al. identified specific subunits of Complex IV that are regulated by HIF-1, which will discussed later [131].

The combined efforts of Complexes I-IV of the ETC pumps electrons from the mitochondrial matrix, which has a low [H^+], to the intermembrane space, which has a high [H^+]. This creates an electrical gradient across the inner mitochondrial membrane and the free energy resulting from this is the proton-motive force [92]. The proton-motive force powers Complex V, otherwise known as ATP synthase, to synthesise ATP from ADP and inorganic phosphate. Complex V is the most complex structure in the inner mitochondrial membrane and is made up of two functional subunits, F_0 and F_1. The F_0 subunit forms the proton channel, where protons from the intermembrane space are translocated back into the mitochondrial matrix, providing the energy required to synthesise ATP. The synthesis of ATP is carried out by the F_1 subunit using the energy generated by the F_0 subunit [93].

1.7 The triggering and amplifying pathways

Pancreatic β-cells secrete insulin via two signalling pathways; the triggering and the amplifying pathways (Figure 1.6). The classical triggering pathway of insulin secretion in the β-cell has been extensively studied and is reasonably well understood [85, 132, 133]. Circulating blood glucose is taken up into β-cells by a glucose transporter called GLUT2 and is metabolised by the glycolytic pathway in the cytosol (Figure 1.2). The glucose sensing enzymatic step is catalysed by the hexokinase glucokinase. Together with the TCA cycle and the oxidative phosphorylation process located in the mitochondrion, ATP is formed resulting in an increase in the cellular ATP:ADP ratio and closing of the ATP-sensitive potassium channels (K_{ATP} channels) [85, 132, 134]. The cell membrane then becomes depolarised, leading to the opening of the voltage dependent Ca^{2+} channels (VDCC) [135, 136]. An increase in cytoplasmic Ca^{2+} concentration finally leads to the secretion of insulin [137] (Figure 1.6).

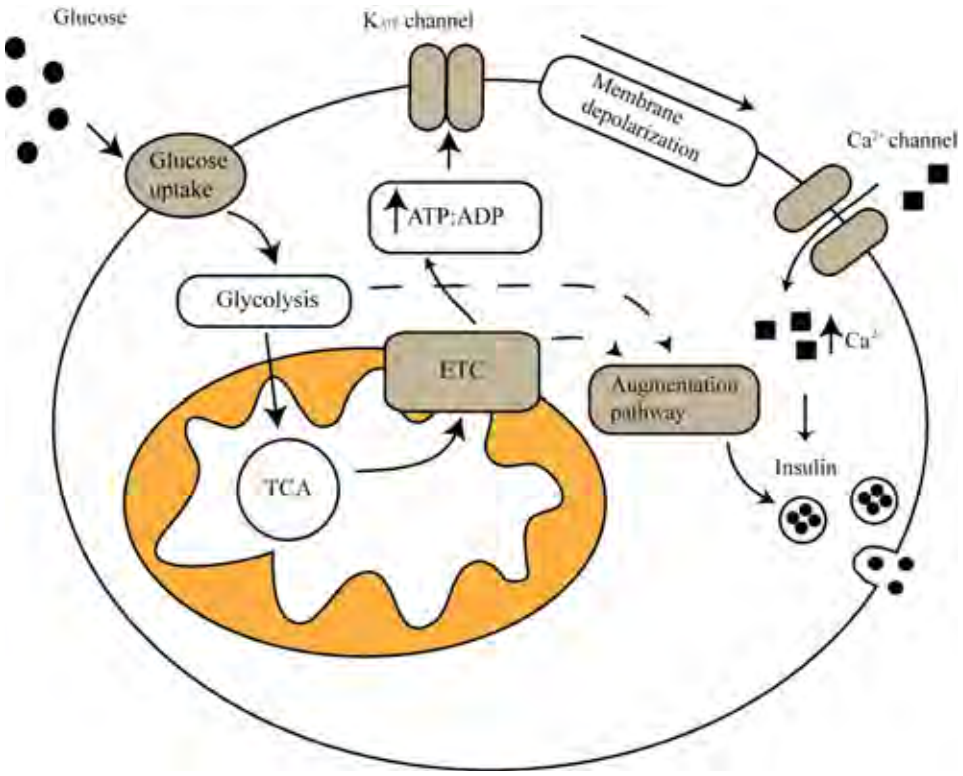

Figure 1.6 Model of insulin secretion in the β-cell. Glucose is taken up and broken down by glycolysis. This provides substrates for the TCA cycle, which in turn provides substrates for the ETC. ATP generation for the ETC increases the ATP:ADP ratio leading to closure of the K_{ATP} channels. Calcium channels open after membrane depolarisation leading to an increase in intracellular calcium and subsequent insulin exocytosis. The amplification pathway of insulin secretion is less well understood but operates independent of changes in the ATP:ADP ratio and the K_{ATP} channels.

The amplifying pathway was identified in 1992 by two independent groups [138, 139]. These experiments showed that glucose stimulation of insulin secretion was still possible even when K_{ATP} channels were either closed (with potassium chloride (KCl)) or kept open (with diazoxide), indicating that another pathway must exist. The exact mechanisms of the amplifying pathway are still yet to be fully understood but insulin secretion via this pathway seems to involve signalling molecules other than ATP:ADP. Excellent reviews regarding the amplification pathway of insulin secretion have been published [132, 133, 140]. Pyruvate from glucose metabolism can be shuttled off via pyruvate carboxylase to form other intermediates which can trigger insulin secretion without the need for closure of K_{ATP} channels. These include NADPH from the malate-pyruvate shuttle and lipid signalling molecules from the malonyl-CoA/LC-CoA pathway [132].

The triggering and amplifying pathways of insulin secretion are not mutually exclusive but complement each other. However, depending on which phase of insulin is occurring, one of the pathways may play a more significant role. It is generally accepted that the first phase insulin secretion is initiated by the triggering pathway and this has much to do with the timing of the event. As stated earlier, first phase insulin secretion occurs within a very short time *in vivo*. It has been suggested that the signalling involved in the amplification pathway may be too slow for this time-frame and thus the triggering pathway is probably the major initiator of first phase insulin secretion [85, 133]. However, a previous study showed that both the triggering and amplifying pathways are involved [141]. This study examined the different signals in the triggering and amplification pathways of insulin secretion and showed that there are shifts in the cytosolic calcium concentration and insulin response curves in first phase insulin secretion, shifts normally associated with the amplification pathway [141].

1.7.1 The role of glycolysis and mitochondrial oxidative phosphorylation in insulin secretion

Mitochondrial oxidative phosphorylation plays an important role for insulin secretion, as it provides much of the needed ATP to change the ATP:ADP ratio [142-144]. It has been clearly established that mitochondrial dysfunctions can lead to diabetes [145-147]. Recent experiments have shown that mice deficient in the pancreatic duodenal homeobox 1 (*Pdx1*), a β-cell master gene, displayed defective insulin secretion due in part to suppression of the mitochondrial transcription factor A (*Tfam*) [145]. *Tfam* is crucial for the stability and transcriptional activity of mitochondrial DNA [148].

However, the question of the relative contribution of mitochondrial oxidative phosphorylation versus other pathways in physiological insulin secretion remains controversial [149-153]. One such argument is in regards to reactive oxygen intermediates (ROI), which include hydroxyl radicals, hydrogen peroxide, superoxide, and singlet oxygen produced in the mitochondria [154], peroxisomes [155], and plasma membrane-associated NAD(P)H oxidases [156]. Excess ROI levels lead to suppression of insulin secretion [157, 158], however, absent or very low ROIs also inhibit insulin secretion. Interesting, low but not excessive ROIs stimulate insulin secretion [157, 159].

While the fact the mitochondrial derived ROI's having a role in insulin secretion is not disputed, some researchers propose that other sources of ROI's may contribute to a larger extent towards insulin secretion than previously thought. Rotenone, an inhibitor of mitochondrial Complex I, stimulates insulin secretion at basal glucose levels [160] and taken together with a previous study showing that rotenone inhibition of mitochondrial activity reduces mitochondrial ROI production in cardiac cells [161] may indicate that extra mitochondrial sources of ROI are responsible for the increased insulin secretion.

1.8 Regulatory genes

Identifying genes that play a role in insulin secretion is an important research area which has the potential to lead to targets for the treatment of diabetes. A summary of some of these regulatory genes is outlined below.

1.8.1 Glucose transporter 2

To perform its biological function, glucose needs to be transported across the plasma membrane and this is facilitated by a family of glucose transporters; GLUT1-12. The members of the GLUT family are highly related but they differ in their tissue specificity and function [162]. GLUT2 is highly expressed in the liver and pancreatic β-cells and is an important part of the glucose sensing mechanism necessary for insulin secretion due to its low affinity and high K_m for glucose [163]. Reduced expression of *Glut2* has been found in animal models of T2D including the neonatal streptozocin rat [164], the diabetic Zucker rat [165], and the db/db mouse [166]. Mice that have a homozygous deletion of *Glut2* display characteristics of T2D, including a loss of first phase but preserved second phase insulin secretion [167]. The relative importance of GLUT2 in humans is less clear. People with some mutations in *GLUT2* develop Fanconi-Bickel syndrome, which is associated with glucose intolerance and diabetes [168]. A V197I mutation in human *GLUT2* has been shown to abolish glucose transport [169] and thought to be associated with T2D [170]. However, in a small sample of human control and diabetic subjects, no change in *GLUT2* mRNA or protein expression was observed [171, 172] and a recent study has suggested that GLUT1 and GLUT3 are the main glucose transporters in human pancreatic β-cells [173]. Indeed, *GLUT1* expression in human islets was higher than *GLUT2* [172]. Therefore, despite the very clear effects of *Glut2* disruption in mice with regards to insulin secretion, human GLUT2 may not have the same roles as it does in rodents.

1.8.2 Glucokinase

β-cells secrete insulin in response to increases in blood glucose levels and thus they need to be able to sense changes in glucose concentrations. GCK is the initial rate determining step of the glycolytic pathway, phosphorylating glucose to glucose 6-phosphate. GCK is thought to be the main glucose sensor for β-cells due to its high K_m of approximately 10 mM and high specificity for glucose [174]. This is supported by the fact that heterozygous mutations in *GCK* cause maturity onset diabetes of the young (MODY). *GCK* mutations account for 50 % of cases of MODY [96-98]. In humans, mutations in *GCK* cause a right-shift in the GSIS curve.

A number of studies have been performed to identify the role of GCK in β-cells [174-179]. Mice with homozygous deletions of *Gck* are born diabetic and die shortly after birth. However, mice with one functioning allele of *Gck* ($Gck^{+/-}$) survive but are hyperglycaemic and display impaired first and second phase glucose stimulated insulin secretion (GSIS) [174, 176]. This was shown to be caused by the inability of glucose to close the K_{ATP} channels and thus inadequate depolarisation of the β-cell [178]. Mice with targeted deletion of *Gck* only in the β-cells display a very similar insulin secretory profile to that of the $Gck^{+/-}$ mice [175]. Interestingly, as *Gck* is only expressed in the liver and pancreatic β-cells, rescue of $Gck^{-/-}$ mice was possible by overexpressing *Gck* in the β-cells only, suggesting that β-cell GCK was more important than liver GCK for glucose homeostasis and survival [174].

1.8.3 K_{ATP} channels

An important step in the triggering pathway of insulin secretion is the closure of the K_{ATP} channels, allowing for subsequent membrane depolarisation and insulin exocytosis. The K_{ATP} channels in general are comprised of a K^+ inward rectifier (Kir6.1 or Kir6.2) and a sulfonylurea receptor (SUR1 or SUR2), with SUR1 and Kir6.2 making

up the pancreatic β-cell K_{ATP} channels [180]. It has been shown that disruption of either of these subunits leads to impaired insulin secretion in mice [181, 182] but conversely leads to hypoglycaemia and increased insulin secretion in people [183-185].

Both *Sur1*$^{-/-}$ and *Kir6.2*$^{-/-}$ mice have been generated and display similar phenotypic changes [181, 182, 186, 187]. GSIS, both first and second phase, was impaired in the *Sur1*$^{-/-}$ and *Kir6.2*$^{-/-}$ mice. Interestingly, euglycaemia was maintained by these animals, as the *Kir6.2*$^{-/-}$ mice had no deviations in their glucose tolerance compared to control littermates [182], and the *Sur1*$^{-/-}$ had normal random fed glucose levels (albeit with slightly impaired glucose tolerance) [181]. This was achieved through different mechanisms. The *Kir6.2*$^{-/-}$ mice was able to maintain normal glucose tolerance in the face of reduced first and second phase GSIS due to an increased insulin sensitivity in peripheral tissue [182]. However, the *Sur1*$^{-/-}$ mice did not display any changes to insulin sensitivity and the authors postulated that euglycaemia was maintained in a fed state by insulin release via K_{ATP} channel independent pathways [181].

1.8.4 Fructose-1,6-bisphosphatase

Fructose-1,6-bisphosphatase is a gluconeogenic enzyme that catalyses the conversion of FBP to F6P. The expression of *Fbpase* has been shown to be increased in a number of diabetic mouse models including the New Zealand Obese (NZO) and the BTBR mice [117, 188] and this increase in expression may be induced by an increase in fatty acids [189, 190]. Insulin secretion was reduced in mice and the MIN6 cell model with an overexpression of *Fbpase* and it is physiologically relevant to human T2D as human T2D islets have an increase in *FBPase* expression [190]. *Fbpase* overexpressing MIN6 cells had reduced glucose utilisation and metabolism, resulting in a reduction in ATP generation, all of which contribute to the observed reductions in insulin secretion [190].

1.8.5 Insulin receptor

The insulin receptor (IR) is a ubiquitously expressed cell surface protein capable of binding insulin with high affinity, setting off a cascade of insulin signalling reactions in peripheral tissues necessary for glucose homeostasis [191]. In humans, heterozygous mutations in *IR* results in syndromes of severe insulin resistance, including Rabson Mendenhall syndrome and leprechaunism, with traits including growth retardation, acanthosis nigricans, and diabetes [192]. The phenotype is very severe in mice with homozygous *Ir* deletion in that they die shortly after birth (48 – 72 hrs) due to diabetic ketoacidosis [193]. Tissue specific *Ir* knockout mice have been made. Muscle specific *Ir* knockout mice display some of the characteristics of T2D, including increased triglyceride and serum free fatty acids but glucose tolerance remains normal without additional insults [194]. More interestingly, mice with β-cell specific knockout of *Ir* display many of the characteristics of T2D, including a loss of first phase but retained second phase insulin secretion [195]. This is of particular importance as it provides a model for T2D development, whereby insulin resistance at the β-cell level (replicated in mice with a β-cell specific knockout of *Ir*) leads to reduced first phase insulin secretion, a major characteristic of disease development [195].

1.8.6 Nicotinamide nucleotide transhydrogenase

Nicotinamide nucleotide transhydrogenase (NNT) is a nuclear encoded mitochondrial protein responsible for the reduction of $NADP^+$ by NADH and conversion of NADH to NAD^+. This makes it particularly important in terms of insulin secretion because it generates NADPH and thus affecting mitochondrial metabolism [196]. First identified to have a role in insulin secretion by Toye et al [197], *Nnt* expression was shown to be significantly lower in C57BL/6J mice, a strain of mice exhibiting impaired glucose homeostasis independent of obesity (including reduced first phase insulin secretion after

feeding). In addition, C57BL/6J mice display a 5-exon deletion in *Nnt*, which is not present in other strains of mice including the closely related C57BL/6N [197, 198]. However, only a few strains of the C57BL/6 mice carry this mutation and subsequent impairments in glucose homeostasis. Regardless, this was subsequently validated by a study showing that mice with mutant forms of *Nnt* have reduced glucose tolerance and first phase insulin secretion, with an accompanied increase in glucose utilisation and decreased ATP production [196]. This phenotype was attributed to ROS mediated activation of UCP2 and thus uncoupling of mitochondrial oxidative phosphorylation [196]. In addition to this, upregulation of *Nnt* was shown to be a cause for insulin hypersecretion in the DBA/2 mice, a diabetes susceptibility mouse model [199].

1.8.7 Nicotinamide phospho-ribosyl-transferase and the Sirtuin family

Recent findings have implicated the enzyme nicotinamide phospho-ribosyl-transferase (NAMPT) and NAD biosynthesis in insulin secretion and metabolism [200, 201]. The enzyme NAMPT is the rate limiting step in NAD biosynthesis. Mice with a heterozygous deletion of *Nampt* have been shown to have impaired glucose tolerance. Isolated islets from *Nampt*$^{+/-}$ mice have reduced GSIS at 15 minutes after stimulation, a time point representing first phase insulin secretion in *in vitro* studies [201]. The authors found that the deficiency in insulin secretion was due to the defects in nicotinamide mononucleotide (NMN) and NAD biosynthesis, possibly altering glycolysis or lipid oxidation as a downstream consequence [201]. The deficiency in NAD would have downstream effects on NAD-dependent enzymes, in particular, the sirtuin family.

The sirtuins are a family of deactetylases and mono-ADP-ribosyltransferases, of which there are seven in mammals, that use NAD as a substrate [202]. It has been previously reported that Sirt1 regulates insulin secretion by repressing *uncoupling*

protein 2 (*Ucp2*) in β-cells [203] and over expressing *Sirt1* in β-cells improves first phase insulin secretion [204]. More recently, Sirt4 has also been implicated as having a role in metabolism. Sirt4 is a mitochondrial protein that has been shown to regulate insulin secretion by repressing glutamate dehydrogenase activity, thereby reducing the ability of mitochondria to generate ATP from glutamate and glutamine [205, 206]. The generation of ATP is vital for the triggering pathway of insulin secretion and thus Sirt4 could play an important role in first phase insulin secretion. These are the only members of the sirtuin family which have been directly associated with insulin secretion although emerging evidence may shed some light on the role of Sirt3 in this respect. Reduced expression of Sirt3 in streptozotocin induced diabetic mice has been reported [207].

1.8.8 Uncoupling proteins

The uncoupling proteins function by dissipating the energy from glucose/lipid oxidation as heat rather than flowing through the electron transport chain to produce ATP [208]. The uncoupling protein UCP1 is the classic and most well known UCP out of five (UCP1 – UCP5) but is predominantly expressed in brown adipose tissue [209, 210]. Of the other UCP's, UCP2 has a definite role in insulin secretion, however research has shown conflicting results. On one side of the argument, *Ucp2* expression has been proposed to be a major factor in obesity, β-cell dysfunction, and T2D, negatively regulating insulin secretion [211]. In clear contrast to this, a more recent study reported that over-expressing *Ucp2* in mice had no effect on insulin secretion [212]. Another study has shown that mice with homozygous knockout of *Ucp2* have impaired β-cell function, possibly due to increased oxidative stress [213]. Therefore, even though UCP2 may have a role in insulin secretion, there are both positive and negative impacts in the current literature.

1.8.9 Disallowed genes

Due to the highly specific nature of β-cells and their ability to secrete insulin, a subset of genes has been described that has been specifically disallowed or repressed to allow the maintenance of glucose homeostasis in the most efficient manner. The first of these genes described were *LdhA* and *Slc16a1*, encoding for lactate dehydrogenase A and monocarboxylic acid transporter 1 respectively [144]. The repression of these genes allow for the maximum amount of ATP to be produced by the β-cell and thus increasing the efficiency of insulin secretion [214]. Two papers have been recently published describing genes that are actively repressed in cells, with one paper specifically for β-cells [215, 216]. Of particular note is the abundance of genes involved in mitochondrial metabolism, some of which could be targets for hypoxia inducible factor-1 (as will be discussed later). From these studies, we now understand that proper β-cell is a function of both the genes that are expressed and the ones that are actively disallowed.

1.9 Oxygen sensing and Hypoxia Inducible Factor-1

Eukaryotes in general are not tolerant to the absence of oxygen, as the reduction of molecular oxygen to water via the electron transport chain provides the energy required to drive the many functions of higher organisms. However, conditions where oxygen is not freely available do occur and an organism must be able to tolerate these adverse conditions or perish. In order to react to adverse conditions such as a lack of oxygen, an organism must have specific oxygen sensing mechanisms in place. In mammals, the oxygen sensor that initiates cellular responses to anaerobic conditions has been described as HIF-1.

HIF-1 is a transcription factor that was first identified in relation to erythropoietin regulation in 1992 [217]. In this study, Semenza and Wang described a 50 nucleotide

sequence in the erythropoietin gene which binds to HIF-1. This sequence was denoted as the hypoxia response element and subsequent studies have shown that the HIF-1 binding site sequence can be represented by the DNA motif 5'-RCGTC-3' [110].

The transcription factor HIF-1 is a heterodimer composed of an α and β subunit, both of which are part of the basic helix-loop-helix Per-ARNT-Single minded family (bHLH-PAS) of proteins. The structure of these proteins (bHLH) enables recognition of DNA binding motifs in target genes while the PAS domain, a sequence of approximately 300 amino acids, functions with the bHLH domain to mediate dimerisation between two proteins [218]. The bHLH-PAS family consists of two major classes of proteins, class A and class B. Class A proteins are ubiquitously expressed and form heterodimers with class B proteins, while class B do the same with class A proteins but do not interact with other class B proteins.

The β subunit of HIF-1 is also called the aryl hydrocarbon nuclear receptor translocator (ARNT or HIF-1β) while the α subunit can be one of three closely related class B proteins denoted as HIF-1α, HIF-2α, and HIF-3α [219]. This review will focus primarily on ARNT and HIF-1α as it has been shown to have the most relevance to energy homeostasis and diabetes.

1.9.1 Aryl hydrocarbon nuclear receptor translocator

The ARNT protein is a ubiquitously expressed protein that acts as a general partner for the class B members of the bHLH-PAS family. First identified in 1992 for its role in binding with the aryl hydrocarbon (dioxin) receptor and mediating carcinogenic effects [220], ARNT has since been shown to participate in many other transcriptional activities. Other heterodimeric ARNT complex functions include developmental regulation of the mouse central nervous system (by partnering with SIM-1 and 2; [221] and binding to HIF-1α to mediate a wide range of responses to hypoxia, which will be

discussed later [222]. It was previously thought that *Arnt* was ubiquitously and constitutively expressed, but it has since been shown that the carbohydrate-responsive element-binding protein (CHREBP) is a negative regulator [223].

Because of the many binding partners of ARNT and its transcriptional role for a number of genes, it is not surprising that ARNT is essential for whole organism survival and development. Studies with embryonic stem cells with a homozygous knockout of *Arnt* (*Arnt*$^{-/-}$) had defective angiogenesis and development, together with a cellular failure to respond to glucose and low oxygen levels. As such, these *Arnt*$^{-/-}$ embryonic stem cells did not survive past embryonic day 10.5 [224]. In a similar study, *Arnt*$^{-/-}$ mice were produced but no embryos survived past gestation day 10.5. This was largely attributed by the authors to be defective vascularisation of the placenta [225].

1.9.2 Hypoxia inducible factor 1α

HIF-1α is a 120 kDa protein and was first described and cloned in 1995 by Wang and Semenza in an attempt to characterise HIF-1 [222]. Since that time, numerous studies have been conducted regarding HIF-1α, gathering evidence detailing its importance in transcriptional events in response to hypoxia, as well as implicating its involvement in diseases such as cancer and diabetes. Unlike ARNT, HIF-1α is not ubiquitously expressed, with mRNA expression only observable in certain organs such as the liver, heart, brain, muscle, kidney, lung, white adipose tissue, and the spleen [226]. Homozygous knockouts of HIF-1α have been made but like *Arnt*$^{-/-}$ mice, *Hif-1α*$^{-/-}$ mice are embryonic lethal, with the embryos having impaired yolk sac vascularisation and abnormal neural development [227].

Oxygen sensing and transcriptional activity of HIF-1 is mainly determined by the activity of the HIF-1α protein. The HIF-1α protein is regulated post translationally in an oxygen dependent manner; under hypoxic conditions, HIF-1α is stable and can

bind to ARNT to commence transcription of target genes (Figure 1.7). However, under normoxic conditions, HIF-1α is targeted for degradation via an oxygen sensitive ubiquitin-proteasome pathway and thus less HIF-1 is transcriptionally active (Figure 1.8; [219]. The degradation of HIF-1α under normoxic conditions begins with the hydroxylation of specific residues within the HIF-1α protein, specifically Pro^{564} and Asn^{803} by prolyl and asparaginyl hydroxylases respectively [228, 229]. These hydroxylases function differently but both require oxygen and iron to function and explains the oxygen sensing nature of HIF-1. The hydroxylated proline residue increases the affinity of HIF-1α for the von Hippel-Lindau tumour suppressor protein (VHL), the recognition and binding component of the pVHL-elonginB-elonginC (VBC) complex. Once VBC is bound, HIF-1α is targeted for polyubiquitination and subsequent proteasomal degradation [228]. A number of studies have found that mice with homozygous pancreatic or β-cell specific knockout of *Vhl* have impaired insulin secretion and significantly impaired glucose tolerance [230-233].

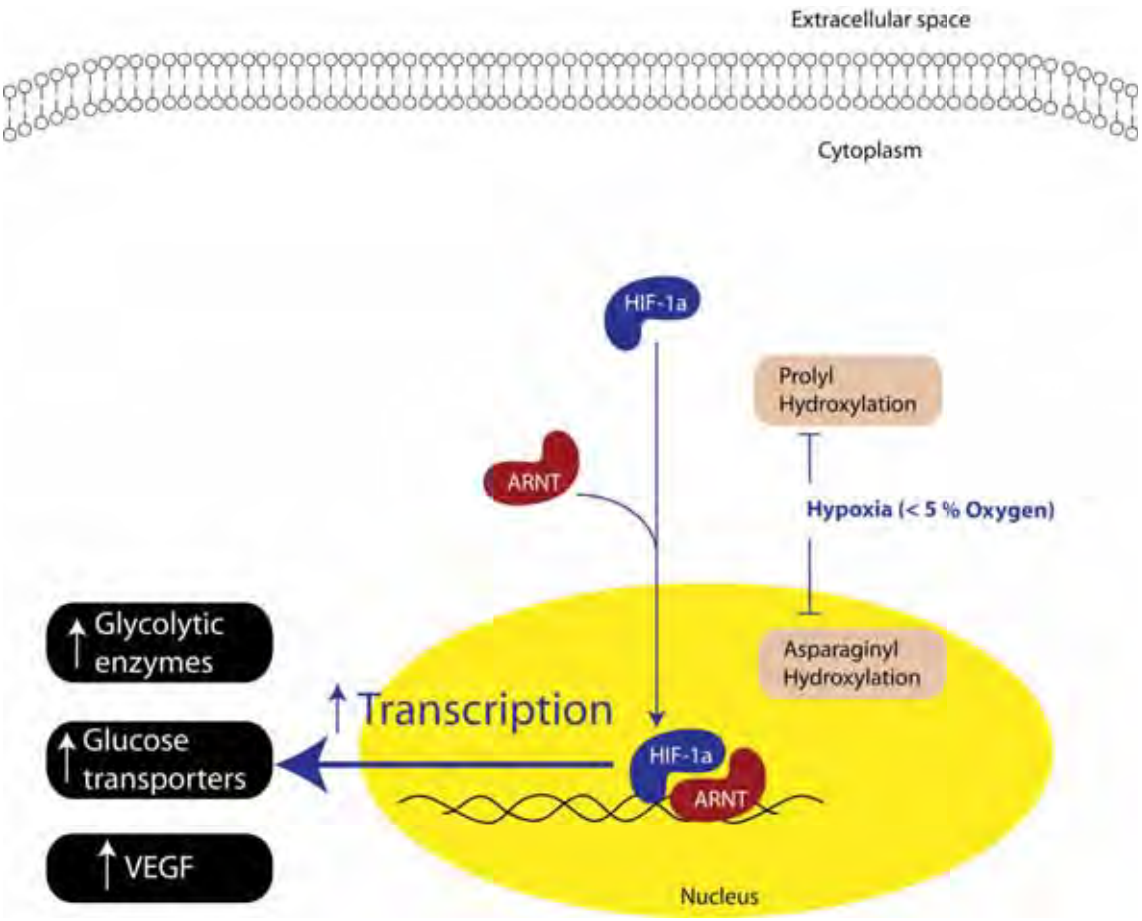

Figure 1.7 HIF-1α regulation under hypoxic conditions. HIF-1α, shown in blue, is regulated in an oxygen dependent manner. In hypoxic conditions (< 5 % oxygen), oxygen dependent prolyl and asparaginyl hydroxylases are not functional and thus HIF-1a is not hydroxylated. HIF-1α is able to bind to ARNT and increase transcription of target genes in the nucleus.

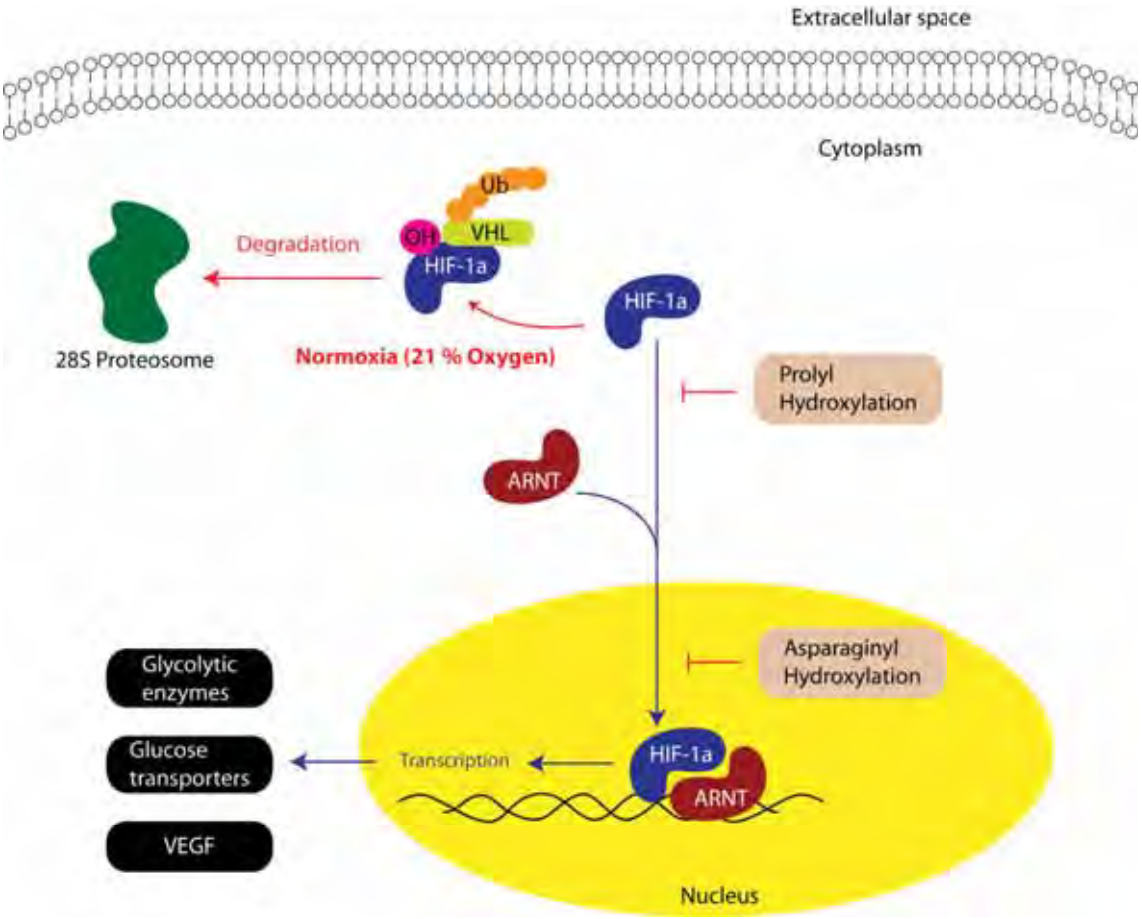

Figure 1.8 HIF-1α regulation under normoxic conditions. HIF-1α, shown in blue, is regulated in an oxygen dependent manner. In normoxic conditions (21 % oxygen), oxygen dependent prolyl and asparaginyl hydroxylases hydroxylate specific proline and asparagine residues on HIF-1α. Hydroxylated proline residues increases the affinity of HIF-1a to the VHL protein. HIF-1α is subsequently ubiquinated and degraded by the 28S proteasome. Asparaginyl hydroxylation inhibits recruited of p300, a necessary co-factor for transcription. Transcription of target genes do not cease as some HIF-1α proteins escape degradation.

However, mice with β-cell specific knockout of both *Vhl* and *Hif-1α* reversed the *Vhl* knockout phenotype and had normal insulin secretion, showing that the massive increase in HIF-1α protein was responsible for the deleterious effects [230]. In addition to this, it has been shown that HIF-1α is a mediator for cytokine induced β-cell dysfunction and that knockdown of HIF-1α partially prevents cytokine-induced inhibition of β-cell apoptosis [234].

Asparaginyl hydroxylation, or factor inhibiting HIF (FIH), provides a secondary oxygen regulated mechanism to inhibit HIF-1 for those HIF-1α proteins that escape the ubiquitin-proteasome degradation pathway. The hydroxylated asparagine residue prevents association with p300, a necessary co-activator for HIF-1 mediated transcription, and thus transcription of target genes is greatly reduced [229]. Mice lacking FIH ($Fih^{-/-}$) have been generated recently and when these mice were place on a high fat diet (HFD), they actually had decreased weight gain and improved insulin sensitivity compared to control mice [235]. This would suggest that FIH may have an important role in regulating metabolism.

Stabilisation of HIF-1α protein under normoxic conditions can be achieved by using an iron chelator such as deferoxamine (DFO) or an iron competitor such as cobalt chloride to inhibit the actions of the hydroxylases [236, 237]. As mentioned previously, both the prolyl and asparagines hydroxylases are dependent on iron to function and therefore, compounds that chelate or outcompete the iron inhibits the activity of these hydroxylases [238] (Figure 1.9). In addition to this, DFO was shown to stabilise HIF-1α protein activity via the cyclooxygenase-2 signalling pathway in colon cancer cells [239]. The use of DFO and its oral analogue, deferasirox (DFS), will be examined in greater detail as one of the aims in this project.

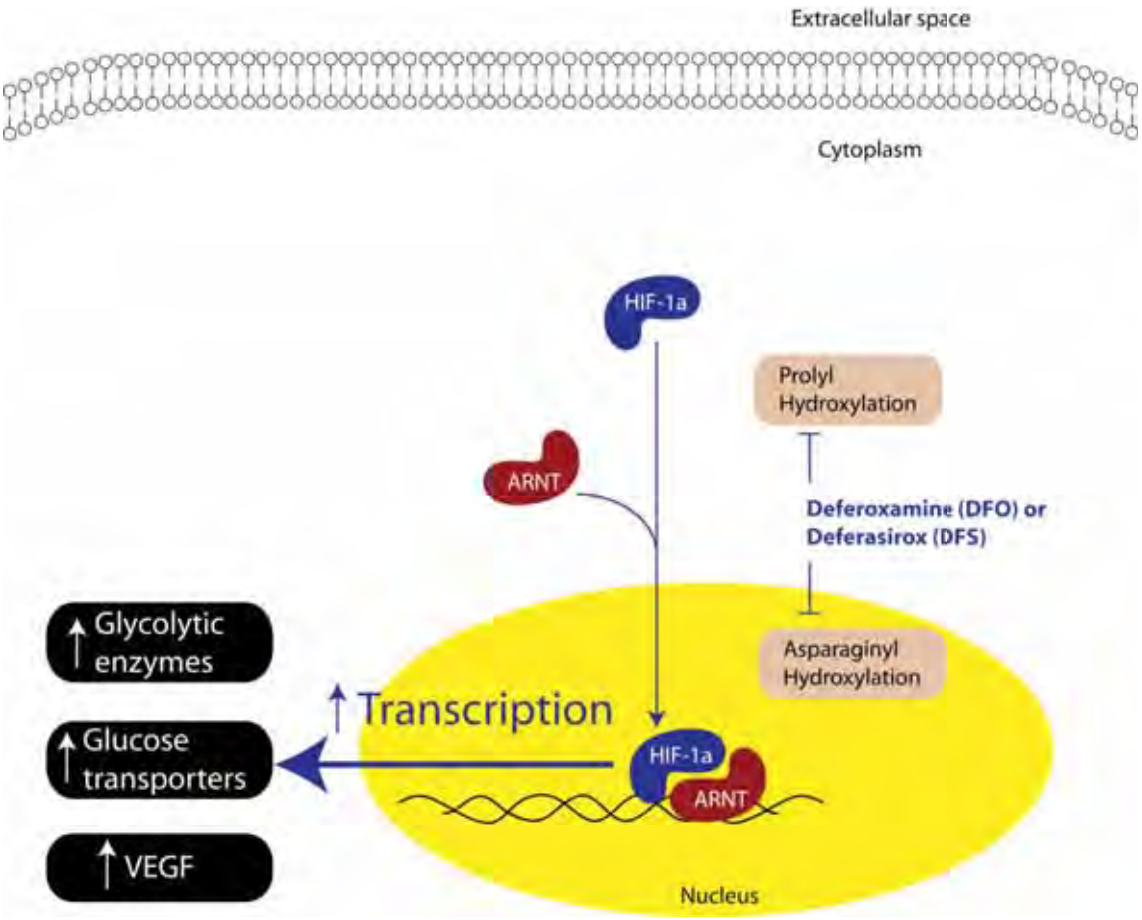

Figure 1.9 Iron chelator regulation of HIF-1α. Prolyl and asparaginyl hydroxylases require iron to function. DFO, or its oral analogue DFS, are iron chelators and in this case would mimic hypoxic conditions and prevent HIF-1α from proteasomal degradation.

1.10 Transcriptional activity of HIF-1

As stated above, HIF-1 mediates a number of transcriptional activities to aid cellular survival under hypoxic conditions. Among the earlier genes identified to be regulated by HIF-1 include *EPO* and the *vascular endothelial growth factor* (*VEGF*), both of which increase delivery of available oxygen to cells under hypoxic stress [217, 240]. However, as oxygen availability declines, there needs to be a transition from oxidative phosphorylation to glycolysis as the principal mechanism of ATP production. It was noted that HIF-1 increases the expression of a number of genes encoding glycolytic enzymes including aldolase A, LDH-A, PGK-1, and enolase 1 [110]. It was also previously thought that mitochondrial respiration decreases due to a reduction in available oxygen but recent evidence seems to indicate that HIF-1 may be actively downregulating mitochondrial oxygen consumption under hypoxic conditions. As described above, PDK1 is an enzyme that regulates the PDC and thus the flow of pyruvate in the TCA cycle. Activation of HIF-1 decreases the expression of PDK1, effectively reducing the flow of pyruvate into the TCA cycle, which in turn will reduce the amount of substrates required for oxidative phosphorylation [241]. Together with an increase in conversion from pyruvate to lactate by LDH-A, evidence now suggests that HIF-1 is regulating mitochondrial respiration in oxygen dependent manner [242].

1.10.1 ARNT and β-cell function

The β subunit of HIF-1, ARNT, has been reported to have an important role in β-cell function [172]. Gunton and colleagues [172] were the first to observed that *ARNT* was markedly reduced in islets of T2D patients. Mice with β-cell specific knockout of *Arnt* had reduced glucose tolerance and absent first and impaired second phase GSIS both *in vivo* and in isolated islets. This was supported by evidence in short interfering RNA (siRNA) knock down of *Arnt* in the MIN6 cell culture line, which showed similar

results. The reduced insulin secretion was accompanied with decreased expression of *glucose-6-phosphoisomerase (G6pi)* and *aldolase (Aldo)*, two key glycolytic genes [172]. The α subunit, HIF-1α, may also have a role in β-cell function and insulin secretion due to the studies performed in mice lacking *Vhl* (as described in Section 1.8.2). The aim of this project is to identify the role of HIF-1α in glucose homeostasis and will be discussed in further detail later.

1.11 Treatments for T2D

First phase insulin secretion is crucial in maintaining glucose homeostasis and a reduction in this early phase may be the first detectable signs of β-cell dysfunction and increased risk of T2D. Therefore, restoration of first phase insulin secretion may have a positive impact on disease development. A study in dogs showed that restoration of first phase insulin secretion (even in the absence of second phase) reduced EGP [66]. As the inability to suppress EGP can lead to IGT [64-66], restoration of first phase insulin secretion could be an important first step in the treatment of T2D.

1.11.1 Lifestyle changes and drug intervention

As the onset of T2D is caused in a large part by obesity and a sedentary lifestyle (as discussed in Section 1.3.1.1 and Section 1.3.1.2), the most obvious treatment would be to make changes in the lifestyle of the patient. Weight loss, by decreasing calorie intake, has been shown to reduce blood glucose to near normal ranges with only a moderate decrease in weight of 2.3 – 4.5 kg [243]. This, coupled with an increase in physical activity, may be the treatment option with the lowest risk and potential to gain the highest benefits.

However, when a change in lifestyle is not feasible for a patient, a range of drug therapies are available. The most common drugs for the treatment of T2D are the

sulfonylureas and biguanides. While both types of drugs have the same outcome of reducing glycated haemoglobin ($HbA_{1C)}$, the mechanisms of each are different. Sulfonylureas (such as Glibenclamide) are a class of drugs that stimulate insulin secretion [244] while biguanides (such as Metformin) reduce hepatic glucose production [245]. Lipase inhibitors, such as Orlistat, can also be used to reduce dietary fat absorption [246, 247]. A relatively new class of drugs are the glucagon like peptide-1 receptor (GLP-1R) agonists. The GLP-1Rs are expressed on the β-cells (among other tissues) and when activated by drugs like exenatide, induces insulin release in a glucose dependent manner [248] while also reducing apoptosis and enhanced β-cell survival [249, 250].

Exogenous insulin can also be used to maintain glucose homeostasis. Unlike the drugs mentioned above, insulin at present can only be delivered by an injection and thus provides one major obstacle to its use. Insulin is also associated with weight gain in T2D patients due to decreased basal metabolic rate and glucosuria caused by improved glycaemia [251].

1.11.2 Bariatric surgery

Advances in medical techniques have allowed individuals with morbid obesity and T2D to undergo various forms of bariatric surgery to reduce body weight. In many cases, this alleviates or cures T2D [252-255]. These types of surgery may involve a variety of procedures but the general premise is reducing the size of the stomach by using a medical device, removal of a portion of the stomach, or bypassing parts of the gastrointestinal tract in morbidly obese patients. While weight loss and the control of diabetes after these types of surgeries has been well established [252-255], but surprising findings include restoration of first phase insulin secretion and loss of insulin resistance, often soon after surgery [252, 255, 256]. The exact mechanisms responsible

for this are as yet unknown. It was thought that weight loss alone was not the answer due to the relatively short time frame for improvement in first phase insulin secretion (one month after surgery) [252] and weight loss obtained through dieting was unable to restore first phase insulin secretion in T2D patients [257, 258]. It has been proposed that changes in gastrointestinal hormones, body weight, adipokines, gluco- and lipotoxicity could be the mechanisms for the restoration of first phase insulin secretion post surgery [252]. However, a new study shows that calorie restriction itself, and decreased pancreatic fat content may well be the factors associated with improvement [258]. Researchers in this study showed that patients with T2D that went on a calorie restricted diet (total energy intake of 2.5 MJ (600 kcal)/day)) showed improvements in fasting glucose one week after the diet and first phase insulin secretion was restored after eight weeks on the diet [258]. This may have been due to the decrease in total pancreatic fat observed in the dieting patients and despite the small sample size (eleven T2D patients and nine matched control individuals), provides fascinating insight into the potential mechanisms for the improvements seen in bariatric surgery patients.

1.12 Summary

It is clear that first phase insulin secretion has an important role in maintaining glucose homeostasis. The initial insulin response after glucose stimulus primes the system, suppressing HGP in the liver and readies insulin sensitive tissue to take up the glucose. Previous research has shown loss of first phase insulin secretion is one of the earliest detectable symptoms for T2D onset and emerging research has identified restoration of this phase of insulin secretion may benefit disease sufferers, the most pronounced being bariatric surgery. Research has identified many factors that play a role in first phase insulin secretion, including ATP:ADP ratios, ROS, HIF-1, VHL, and members of the sirtuin family. Therapies which increase first phase insulin secretion include weight

loss and GLP-1R agonists. Additional research will uncover even more regulatory genes and therapies and increase our ability to treat T2D.

1.13 Hypotheses

- Loss of first phase GSIS is due to an underlying defect in metabolic activity including that of ATP generation and glucose metabolism.
- Due to the phenotype observed in mice with β-cell specific knockout of *Arnt*, loss of its partner *Hif-1α* will also cause impaired glucose tolerance and insulin secretion, in particular first phase insulin secretion.
- If the previous hypothesis proves to be correct, then increasing HIF-1α will be beneficial to glucose tolerance and insulin secretion.

1.14 Aims

General aims:

- To identify models of reduced first phase GSIS.

Specific aims:

- Identify the metabolic differences between low and high passage MIN6 cells.
- Identify phenotypic changes in regards to glucose homeostasis in mice with β-cell specific knockout of *Hif-1α*.
- Determine the effects of different methods of increasing HIF-1α protein in both mice and MIN6 cells.

Chapter 2

Materials and methods

2.1 Materials

All chemicals and reagents were obtained from Sigma-Aldrich (Australia) unless otherwise stated.

2.1.1 Buffers and solutions

Acid ethanol

10 mM HCl in 70 % ethanol

Bovine calf serum

Bovine calf serum (BCS, Hyclone, USA) was heat inactivated for 30 minutes at 56 °C before use.

Cell lysis buffer

10 mM	Tris-HCl
1 %	Triton X-100
0.5 %	NP-40
150 mM	Sodium chloride
10 mM	Sodium phosphate
100 mM	Sodium fluoride
1 mM	EDTA
1 mM	EGTA
10 mM	Sodium orthovanadate
10 mM	Sodium orthophosphate

One tablet per 50 ml of Complete protease inhibitor cocktail tablets (Roche, Germany) was added and pH 7.4.

DNA isolation buffer

670 mM	Tris pH 8.8
166 mM	Ammonium sulphate
65 mM	Magnesium chloride
10 %	β-mercaptoethanol
5 %	Triton X-100

Islet culture media

10 %	BCS
50 mM	Hepes
2 mM	L-glutamine

Made up with Roswell Park Memorial Institute-1640 medium (RPMI-1640, Invitrogen, USA).

Krebs buffer

115 mM	Sodium chloride
4.7 mM	Potassium chloride
1 mM	Magnesium sulphate
1.2 mM	Potassium sulphate
25 mM	Sodium bicarbonate
1 mM	Sodium pyruvate
10 mM	Hepes

pH 7.4

Phosphate buffered solution (PBS)

3.6 %	Disodium hydrogen orthophosphate
0.2 %	Potassium chloride
0.24 %	Potassium dihydrogen orthophosphate
8 %	Sodium chloride

Medium 199 + BCS (M199 + BCS)

10 %	BCS
3.3 mM	Sodium bicarbonate

Made up with Medium 199 (M199, Sigma-Aldrich, USA)

MIN6 culture media

10 %	BCS
25 mM	Hepes
2 mM	L-glutamine
285 μM	β-mercaptoethanol

Made up with high glucose (25 mM) Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA).

RIPA cell lysis buffer

0.5 %	Sodium deoxycholate
10 mM	Hepes
1 %	NP-40
0.1 %	SDS

pH 7.4

Serum free DMEM

25 mM	Hepes
2 mM	L-glutamine

Made up with no glucose DMEM (Invitrogen, USA).

Staining solution

2 %	Goat serum
1 %	BSA

Made up with PBS

2.2 In vivo mouse studies

2.2.1 β-*Hif-1α* mice

The β-cell specific *Hif-1α* knockout (β-*Hif-1α*) mouse is a conditional gene knockout mouse generated on a C57Bl/6 background using the Cre-lox system. Cre-lox recombination involves the targeted splicing of a specific DNA sequence using a site specific Cre recombinase in order to create a tissue-specific knockout. Mice with floxed *Hif-1α* were kindly donated by Prof. Gonzalez [259]. These mice had exons 13 - 15 of *Hif-1α* flanked by *lox-p* sites which were introduced into the *Hif-1α* gene by standard gene disruption techniques using embryonic stem cells. Floxed *Hif-1α* mice were interbred with mice expressing *Cre*-recombinase under control of the rat insulin promoter (RIP-*Cre*), to generate mice homozygous for floxed *Hif-1α* and either heterozygous or wild type for RIP-*Cre* (*Hif-1α flox/flox* and RIP-*Cre* positive or *Hif-1α flox/flox* and RIP-*Cre* negative. Only one copy of *Cre* is required to produce the β-cell specific phenotype, thus the phenotype is passed on in an autosomal dominant manner. Mice were inbred C57Bl/6 for more than twelve generations to ensure genetic homogeneity. Importantly, RIP-*Cre* transgenic mice in our colony have no difference in

glucose tolerance compared to either wild type or *Hif-1α flox/flox* controls ($p > 0.6$ for all time points during glucose tolerance testing) [260]. All animal work was approved by, and performed in accordance with, the Garvan Institute of Medical Research Animal Ethics Committee.

2.2.2 Mouse housing

All animals were housed in the Biological Testing Facility at the Garvan Institute of Medical Research, which employs a 12 hour on-off light cycle (0700-1900 on, 1900-0700 off). Mice were housed in standard filtered boxes with sterile bedding.

2.2.3 Diet

2.2.3.1 Standard chow

Mice were provided with standard chow food containing 59.9 %, 26.7 % and 13.4 % calories from carbohydrate, protein and fat respectively (Agrifood technology, Australia) unless otherwise specified and water *ad libitum.*

2.2.3.2 High fat diet

The high fat diet was prepared according to Rodent Diet D12451 from Research Diets Incorporated. The ingredients were as follows:

261 g	Casein
230 g	Sucrose
193 g	Starch
51 g	Bulking mineral mix
14.8 g	Trace minerals (including iron, iodine, zinc, copper, magnesium, manganese, etc),
57 g	Bran

3.4 g	Methionine
23 g	Gelatine
4.6 g	Choline bitartrate
29.6 g	AIN Vitamin Mix 76A (ICN Biomedicals, Australia)
68 g	Safflower oil
500 g	Melted Allowrie lard

Dry ingredients were mixed thoroughly before addition of oil and lard. Water was provided *ad libitum*.

2.2.4 Glucose tolerance tests

Glucose tolerance tests (GTT) were performed on mice after being fasted overnight for 16 hours. Glucose was administered at a dose of 2 g/kg by intraperitoneal injection in the form of a 20 % dextrose solution. Blood glucose was measured via the Accucheck Advantage II glucometer (Roche, Australia) prior to, and at 15, 30, 60, 90 and 120 minutes after the dextrose injection.

2.2.5 Glucose stimulated insulin secretion

GSIS was performed on mice after being fasted overnight for 16 hours. Glucose was given at a dose of 3 g/kg in the form of 20 % dextrose by intraperitoneal injection. Blood (15-20 μl) was collected in a 1.5 ml microcentrifuge tube containing 2 μl of EDTA/proteinase inhibitor cocktail (10 ml of 0.5 M EDTA, 1 Complete proteinase inhibitor cocktail tablet (Roche Diagnostics, USA)) prior to, and at 2, 5 and 20 minutes after the dextrose injection. Blood was immediately centrifuged for 2 minutes at max speed and the supernatant stored at -20 °C for insulin ELISA assay.

2.2.6 Blood collection

Blood was collected by a lateral tail nick 1-3 mm from the end of the tail for all tests unless otherwise specified.

2.2.7 Genotyping

Genotyping for *Cre* and *Hif-1α* was performed by PCR and separation on 1.5 % agarose gels using genomic DNA from 2-4 mm tail tips. Tips were digested overnight in 200 µl DNA isolation buffer with 0.5 µl proteinase K per sample (Roche, Germany) at 65 °C. PCR was performed using standard protocols with 5 µl of GoTaq Master Mix Taq polymerase (Promega, USA), 1 µl of DNA solution, 2 µl of *Cre* or *Hif-1α* primers, and 2 µl of *Irs-2* primers (control). The *Irs-2* primers were included in every *Cre* genotyping reaction as a positive control which confirmed successful DNA amplification for *Cre* negative samples. The sequences of the primers can be found in Table 2.1. The PCR was performed using the following:

Initial melt step	5 minutes at 95 °C
35 cycles	
- Denature	45 seconds at 95 °C
- Anneal	45 seconds at 60 °C
- Extend	45 seconds at 72 °C
Final extension	6 minutes at 72 °C

2.3 Mouse islet isolation

After clamping of the Ampulla of Vater, 3 ml of collagenase (Liberase, Roche, USA) diluted in M199 was injected into the pancreas by bile duct cannulation with a 30 gauge needle. Unless otherwise stated, all centrifugation steps were performed at 4 °C. Each

distended pancreas was digested in a 50 ml falcon tube for 18 minutes in a 37 °C water bath. Tubes were shaken and placed on ice. Cold M199 + BCS was added to a total volume of 50 ml. Tubes were alternately shaken and vortexed for 3 minutes. After centrifugation for 2 minutes at 129 x g, supernatant was decanted, fresh M199 + BCS was added and tubes were vortexed. The contents were sieved, with an extra 30 ml M199 + BCS added. The new tube was centrifuged for 2 minutes at 314 x g and the supernatant decanted. A gradient was formed with 20 ml Ficol 1.077 then 10 ml M199 (without BCS). Tubes were centrifuged for 22 minutes at 1612 x g (1/1 acceleration/deceleration), and the islets were removed from the interface and put into a clean tube. M199 + BCS (50 ml) was added and the tube was centrifuged for 2 minutes at 314 x g. The supernatant was aspirated leaving a 10 ml remnant, M199 + BCS was added to 25 ml to resuspend the pellet and the tube left on ice for 4 minutes. This last step was repeated. M199 + BCS was added to 50 ml. The tube was centrifuged for 2 minutes at 314 x g and the supernatant decanted. The remaining mixture was centrifuged for 2 minutes at 314 x g in a 1.5 ml microcentrifuge tube and the supernatant removed. Isolated islets were used for *ex vivo* assays or snap frozen in liquid nitrogen and stored at -80 °C.

2.4 Maintenance of cell culture

MIN6 cells (originally from Dr Miyazaki [261]) were obtained from Dr. Ross Laybutt (Garvan Institute of Medical Research, Australia) and were routinely maintained in MIN6 culture media in a tissue culture incubator at 37 °C with 5 % CO_2. Media was replaced every second day. MIN6 cells were subcultured by washing with PBS and then incubated with 3 ml of 0.05 % trypsin-EDTA (Invitrogen, USA) for 5 minutes at 37 °C. Cells were centrifuged for 5 minutes at 129 x g and divided into 75 cm^2 flasks. The MIN6 cells presented in this study were performed at passage 30-40 (low passage)

and passage 60-70 (high passage). All assays used MIN6 grown to 70-80 % confluence unless otherwise stated.

2.5 Assays

2.5.1 Cell proliferation assay

Cell proliferation was performed using the FITC BrdU Flow Kit (BD Pharmingen, USA). Cells were pulsed with 10 µl per ml of 5-bromo-2-deoxyuridine (BrdU) solution (1 mM BrdU in PBS) for 40 minutes in MIN6 media at 37 °C with 5 % CO_2. Cells were dislodged by 0.05 % trypsin-EDTA and BrdU and 7-amino-actinomycin D (7AAD) staining was performed as per manufacturer instructions. Flow cytometric data was acquired using a fluorescent automated cell sorting (FACS) Canto (BD Biosciences, USA) and analysed using FlowJo software (Tree Star).

2.5.2 Insulin secretion assay

Media used for insulin secretion was serum free DMEM with no glucose. Glucose was added to prepare desired concentrations and warmed to 37 °C prior to use. Isolated islets and MIN6 cells were treated in the same manner. Cells were washed twice with PBS and basal serum free DMEM was added and cells incubated for 2 hours to equilibrate the cells. The cells were then washed with basal serum free DMEM and placed in 1 mM glucose media and samples taken after 15 minutes for measurement of insulin secretion. The media was then replaced with the next glucose concentration media for 15 minutes and repeated for more glucose concentrations. After completion of the incubations, the cells were lysed with acid ethanol for measurement of total insulin content. Insulin secretion with addition of 1 mM pyruvate, 30 mM L-arginine, or 30 mM KCl was performed after an incubation time of 30 minutes before samples were

taken. Insulin secretion with the addition of 0.0625 μM bromopalmitate was performed after an incubation time of 2 hours before samples were taken.

For insulin secretion time courses, MIN6 cells were grown in 12-well plates and insulin secretion samples was collected as described above for 1 mM glucose. After the addition of 25 mM serum free media, 10 μl was removed at each time point (2, 5, 10, 15, 30, 60 minutes) and total insulin collected as described above. The total incubation volume was 1 ml in all experiments. Insulin was measured by ELISA (Crystal Chem, USA) as per manufacturer's instructions.

2.5.3 Measurement of protein

Protein was measure using the *DC* Protein Assay Kit (Bradford, USA) according to manufacturer instructions.

2.5.4 Measurement of intracellular ATP content

Intracellular ATP content was measured using the ATP Bioluminescence Assay Kit CLS II (Roche, Australia). MIN6 cells were equilibrated as described in 2.5.2 then serum free DMEM was replaced with fresh warm 1 mM serum free DMEM and maintained in an incubator at 37 °C with 5 % CO_2. Glucose was added to make up to 25 mM at each time point. After a total incubation time of 60 minutes, MIN6 cells were placed on ice, washed twice with ice cold PBS, and lysed with cell lysis buffer. Assay was performed according to manufacturer's instructions and measurement of ATP was performed using the TopCount NXT (Packard, USA). Results were corrected for total protein as described in Section 2.5.3.

2.5.5 Glucose oxidation assay

MIN6 cells were grown in 25cm^2 flasks, washed twice with PBS, and equilibrated in Krebs buffer with 1 mM glucose for 2 hours. After equilibration, cells were washed with fresh warm Krebs buffer with 1 mM glucose and media replaced with fresh warm Krebs buffer at 1, 5, 11, or 25 mM glucose concentrations with 0.1 μCi/ml of D-[U-^{14}C]-glucose (GE Healthcare, USA). Filter paper soaked in 5 % KOH was suspended over the cells and the flasks sealed shut. MIN6 cells were incubated at 37 °C with 5 % CO_2 for 1 hr and the reaction stopped by the addition of 500 μl of 40 % perchloric acid. Radioactivity was counted in 4 ml Microscint-20 (Perkin Elmer, USA) using the LS 6500 Scintillation Counter (Beckman Coulter, USA). Results were corrected for specific activity and total protein as described in Section 2.5.3.

2.5.6 Glucose uptake assay

MIN6 cells were grown in 6-well plates and equilibrated as per 2.5.5. After washing, media was replaced with fresh warm Krebs buffer at 1 or 25 mM glucose concentrations with 1 μCi/ml 2-deoxy-[1,2-^{3}H]-glucose (Perkin Elmer, USA). MIN6 cells were incubated at 37 °C with 5 % CO_2 for exactly 5 minutes and reaction stopped by placing on ice and washing twice with ice cold PBS with 5 % glucose. Cells were lysed with 500 μl of modified cell lysis buffer and radioactivity counted as in Section 2.5.5. Results were corrected for total protein as described in Section 2.5.3.

2.5.7 Lipid oxidation assay

MIN6 cells were grown in 25cm^2 flasks, washed twice with PBS, and incubated in Krebs buffer plus 0.25 % fatty acid free BSA (Sigma-Aldrich, USA) with 1 mM glucose at 37 °C with 5 % CO_2 for 2 hours. After equilibration, cells were washed with fresh warm Krebs + BSA buffer with 1 mM glucose and media replaced with fresh warm

Krebs + BSA buffer at 1 or 25 mM glucose concentrations with 0.125 mM palmitate and 0.25 μCi/ml of [1-^{14}C]-palmitic acid (GE Healthcare, USA). Filter paper soaked in 5 % KOH was suspended over the cells and the flasks sealed shut. MIN6 cells were incubated at 37 °C with 5 % CO_2 for 24 hours and the reaction stopped by the addition of 500 μl of 40 % perchloric acid. Radioactivity was measured as described in Section 2.5.5 and results were corrected for total protein as described in Section 2.5.3.

2.5.8 Measurement of lactate

Cells were incubated in serum free DMEM containing 1 or 25 mM glucose for 2 hours and lactate was measured using the BioVision Lactate Assay Kit II (BioVision, USA) according to manufacturer's instructions. Results were corrected for total protein as described in Section 2.5.3.

2.5.9 Measurement of oxygen respiration

MIN6 cells were grown in 10 cm dishes and cells dislodged by incubating with 3 ml of 0.05 % trypsin-EDTA (Invitrogen, USA) for 5 minutes at 37 °C. Oxygen respiration was measured at 37 °C in MIN6 cell culture media in a Clark-type oxygen electrode (Strathkelvin Instruments, Scotland). Maximal respiration was measured in the presence of 100 nM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, Sigma-Aldrich, USA). Results were corrected for total protein as described in Section 2.5.3.

2.6 Gene expression studies

2.6.1 RNA extraction

Isolated islets were homogenised using a QIAshredder biopolymer shredding spin column (Qiagen, USA) but MIN6 cells did not require this step. RNA extractions were

performed using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. RNA was quantitated using the NanoDrop 1000 (Thermo Scientific, USA) and was stored at -80 °C in sterile water.

2.6.2 cDNA synthesis

cDNA was synthesised as per Cheng et al [260] from 1 μg of RNA using random hexamer primers and the Superscript III RT kit (Invitrogen, USA) according to the manufacturer's instructions.

2.6.3 Real time PCR

Real time PCR was performed using Sybr Green PCR Master Mix (Applied Biosystems, UK) as per Cheng et al [260] with an ABI 7900HT thermal cycler (Applied Biosystems, UK). A full list of primers can be found in Table 2.1.

2.6.4 MIN6 cell transfection

MIN6 cells were grown to 60-70 % confluence and washed with PBS. Cells were transfected using Lipofectamine 2000 (Invitrogen, USA) diluted in serum free DMEM supplemented with 25 mM glucose. Gene knockdown was mediated by using ON-TARGET *plus* siRNA (Thermo Scientific, USA) and 2 X the recommended amount of Lipofectamine. Scrambled siRNA (Thermo Scientific, USA) was used as the control in all gene knockdown experiments.

HIF-1α overexpression was mediated by using a dual P402A and P577A mutant construct of HIF-1α cloned into the pcDNA3 vector kindly donated to us by Dr. Yatrick Shah (University of Michigan, USA). Lipofectamine was used at 2 X the recommended concentration but otherwise performed as per manufacturer's instructions. pcDNA3

empty vector was used as the control in all overexpression experiments. Experiments were performed 48 hours after transfection of MIN6 cells.

2.7 Histology and immunohistochemistry

2.7.1 Immunohistochemistry: insulin

Six μm thick sections were cut from paraffin-embedded, formalin fixed pancreas. Six sections were chosen per pancreas, equally spaced throughout the paraffin block. Sections were incubated at 70 °C for 10 minutes prior to de-waxing with xylene and standard progressive ethanol rehydration. Insulin staining was performed using the DakoCytomation EnVision+ Dual Link System-HRP (DAB+) Kit (Dako, USA) as per manufacturer's instructions. Rabbit insulin polyclonal antibody (Cell Signaling Technology, USA) was diluted 1:100 in Antibody Diluent (Dako, USA). After insulin staining, slides were counterstained using a standard haematoxylin and ethanol dehydration protocol. Coverslips were mounted using GVA Mount non-aqueous mounting solution (Zymed, USA).

2.7.2 Immunofluorescence: HIF-1*α*, ARNT, and insulin

Section cutting, de-waxing, and rehydration were performed as described in Section 2.7.1. Antigen retrieval consisted of a pressure cooker treatment for 1 minute at 125 °C followed by 10 seconds at 95 °C in Dako Target Retrieval Solution pH 9.0 (Dako, USA). After a water rinse, sections were blocked for 1 hour at room temperature in staining solution, then incubated overnight at 4 °C with the primary antibody mixture consisting of one or more of the following: mouse monoclonal HIF-1α (Novus Biologicals, USA), mouse monoclonal ARNT (BD Biosciences, USA), and rabbit polyclonal insulin (Cell Signaling Technology, USA), all diluted 1:100 in staining

solution. After three by 5 minute washes in PBS, sections were incubated in the dark at room temperature for 1 hour with a secondary antibody mixture consisting of 1:100 anti-rabbit Cy2 IgG, 1:100 anti mouse Cy3 IgG, and 1:1000 4',6-diamidino-2-phenylindole (DAPI, Dako, USA) diluted in staining solution. After a further three PBS washes, coverslips were mounted using Fluorguard Antifade Mounting Solution (Sigma-Aldrich, USA)

2.7.3 Quantification of β-cell mass

Sections were prepared and stained with insulin as outlined in Section 2.7.1 and slides were scanned at the highest possible resolution setting on a flatbed scanner. Images were analysed using ImageJ (v1.45b) and β-cell mass was determined as the average proportion of positive insulin staining cells to total area of the section. In cases where pancreas weight was known, this was then converted to an average mass.

2.8 Statistical Analysis

For all figures, error bars indicate ± SEM. Unpaired 2-tailed t-tests were used to compare two variables, and ANOVA with post-hoc testing (Bonferoni or Tukey's) was used for multiple comparisons. A p-value of <0.05 was considered significant.

Table 2.1 Primer sequences

Primer	Sequence
Acaa1a	cctgactcctatggggatga cccttgtcatccaggacagt
Acadm	aggtttcaagatcgcaatgg ctccttggtgctccactagc
Akt2	tcactctccatcctcccaac tttgtgttcccttccctgtc
Arnt promoter	gcttcctagctcaggcttcc aagagccactccgcagatta
Cre genotyping	aggtgtagagaaggcacttagc ctaatcgccatcttccagcagg
Echdc3	ccaccgcaagctaagaaaag gtgtcctgaggaagcgactc
Fas	gctgcggaaacttcaggaaat agagacgtgtcaactcctggact
Gck	gagatggatgtggtggcaat accagctccacattctgcat
Glut1	ctggtctcaggcaaggaaag acctatggccaaggagacac
Glut2	catgctgagctctgctgaag acagtccaacggatccactc
Hahd	ccaccagacaagaccgattt tcaatgaggtatggcaccaa
Hif-1α	tcaagtcagcaacgtggaag tatcgaggctgtgtcgactg
Hif-1α genotyping	ctgtcttccctgcttaggtctt gagatggagaaggaggttagt acgttggctcatggtgtacttt
Hnf4α	ggtcaagctacgaggacagc atgtacttggcccactcgac
HMG CoA reductase	agccgaagcagcacatgat cttgtggaatgccttgtgattg

HMG CoA synthase	gccgtgaactgggtcgaa gcatatatagcaatgtctcctgcaa
Insulin 2	tttgtcaagcagcacctttg tctacaatgccacgcttctg
Irs-2 genotyping	gtagttcaggtcgcctctgc ttgggaccaccactcctaag
Ldh-A	ccgttacctgatgggagaga gtaggcactgtccaccacct
Nampt	agatactgtggcgggaattg gctatcgctgaccacagaca
Pfk	atggcaaagctatcggtgtc acacagtcccatttggcttc
Sirt3	aggtggaggaagcagtgaga gcttggggttgtgaaagaaa
Srebp1c	ctcaggagagttggcacctg gagccatggattgcacattt
Tbp	atgatgactgcagcaaatcg atgatgactgcagcaaatcg
Ucp2	tcccctgttgatgtggtcaa cagtgacctgcgctgtggta
Vhl	tgcctaaagcgtggagtctt ctggcctgggctatacaaaa

Chapter 3

Metabolic characterisation of MIN6 cells

3.1 Introduction

T2D is characterised by the inability of beta cells to secrete enough insulin to maintain glucose homeostasis, usually accompanied by insulin resistance: impaired action of insulin on target tissues. Pancreatic beta cells secrete insulin in a biphasic manner, with the first phase occurring very rapidly, commonly defined as within the first 10 minutes after glucose stimulation [262]. In whole body systems, insulin secretion normally peaks at 1-2 minutes after stimulation [61]. Second phase insulin secretion is more gradual and long lasting, typically reaching a plateau 25-30 minutes after stimulation [61] (Figure 1.1).

The classic pathway of insulin secretion is generally well accepted and begins with glucose entry into the cell, and a rise in the ATP:ADP ratio as glucose is metabolised via glycolysis and oxidative phosphorylation [85, 132, 134]. This closes the K_{ATP} channels and depolarises the plasma membrane [135, 136]. This in turn opens the VDCCs, which allows Ca^{2+} to enter into the cell and leads to subsequent insulin exocytosis [137]. Interestingly, patients early in the course of T2D lose first phase but often retain second phase insulin secretion [263]. Impairment of first phase insulin secretion is a predictor of future T1D and T2D risk [52, 264]. Many additional mechanisms regulate insulin secretion including the rate of anapleurosis and changes in glutamate, GTP, and GDP levels [85].

The MIN6 cell line was derived from a mouse insulinoma and is one of a few cell lines that display the characteristics of pancreatic β-cells, including insulin secretion in response to glucose and other secretagogues [261, 265]. It is known that MIN6 cells with high passage numbers begin to lose their ability to secrete insulin [266-268]. High passage MIN6 cells have gene expression changes, including down-regulation of genes such as phospholipase D1 and cholecystokinin [268].

High and low passage MIN6 cells also differ at the protein level, with high passage MIN6 cells having lowered expression of some proteins that are involved with correct protein folding in the ER and antioxidant enzymes for handling of ROS [266]. This is in contrast to studies comparing glucose responsive and unresponsive sub-lines of low passage MIN6 cells, with one study showing no change in GLUT2 expression [269] while another study showing barely detectable GLUT2 expression, even in their glucose responsive MIN6 cell line [270]. These studies showed changes in gene and protein expression, however metabolic changes have not been profiled with a panel of functional assays in detail before.

3.2 Aims and strategies

These studies sought to identify changes which would result in impaired insulin secretion in high passage MIN6 cells. In particular, we focused on the following:

i) Insulin secretion using glucose and non-glucose secretagogues

ii) Metabolic assays including the measurement of intracellular ATP content, glucose uptake, glucose and lipid oxidation

iii) Expression of changes in key glycolytic, lipid handling, and lipid oxidation genes

These assays will characterise the underlying metabolic changes that cause impaired GSIS in high passage MIN6 cells.

3.3 Results

3.3.1 Insulin secretion in low and high passage MIN6 cells

Low passage MIN6 cells displayed a dose response in insulin secretion when stimulated with increasing glucose concentrations. This culminated in an approximate 1.7-fold increase in insulin secretion at 25 mM glucose ($p < 0.05$, Figure 3.1). This response to high glucose was not observed in high passage cells. GSIS time courses were studied and as expected, low passage MIN6 cells secreted increasing amounts of insulin over time (Figure 3.2). Interestingly, high passage MIN6 cells eventually responded to glucose stimulation after 60 minutes at 25 mM glucose, however this was still significantly less than that seen in low passage MIN6 cells ($p < 0.05$, Figure 3.2). Even though that the fold change at later time points was higher, the 15 minute time point was chosen to assess first phase insulin secretion. Thus, high passage MIN6 cells had a complete lack of first phase insulin secretion while retaining some second phase secretion.

3.3.1.1 Insulin secretion in response to non-glucose secretagogues

Non-glucose secretagogues were used to test insulin secretion. KCl treatment at 30 mM induced strong and equivalent insulin secretion in both low and high passage MIN6 cells, demonstrating that high passage MIN6 cells were still able to secrete insulin (Figure 3.3). KCl stimulated insulin secretion in low and high passage cells were significantly greater than with 25 mM glucose ($p < 0.05$, Figure 3.3). L-arginine is a potentiator of GSIS and stimulation was evident in low and high passage MIN6 cells at both 1 and 25 mM glucose ($p < 0.05$, Figure 3.3). High passage MIN6 cells did not achieve levels near those in low passage cells with L-arginine stimulation. KCl concentration was not altered with glucose and L-arginine stimulation.

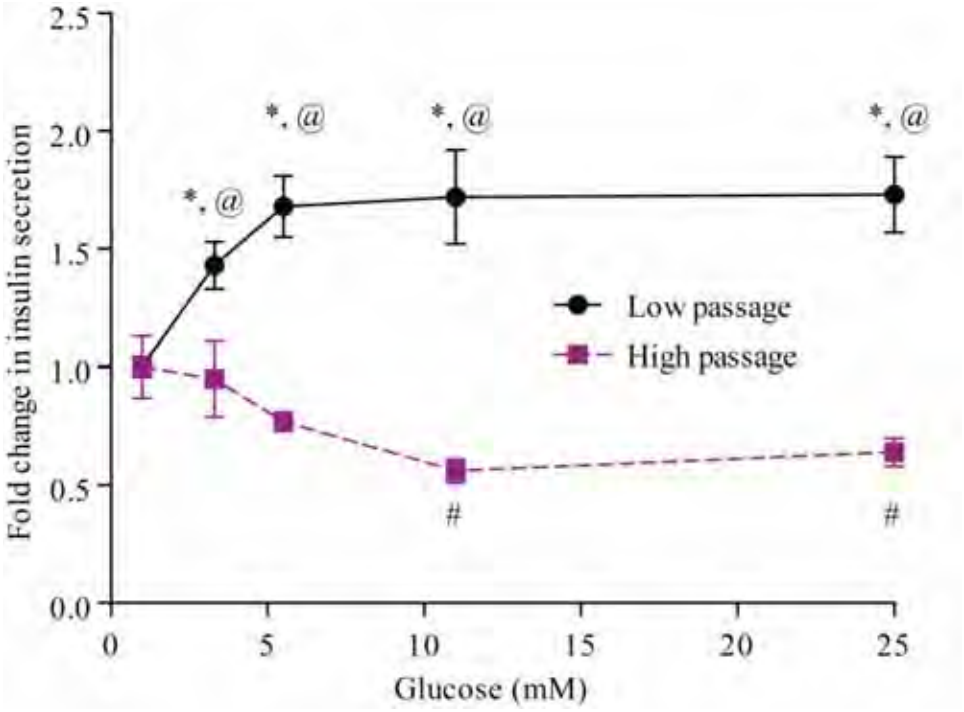

Figure 3.1 GSIS dose response in low and high passage MIN6 cells. Low passage MIN6 cells responded in a dose dependent manner but this was not observed in high passage MIN6 cells (samples taken at 15 minutes after stimulation, representing first phase insulin secretion. * $p < 0.05$ compared to low passage MIN6 cells at 1 mM glucose, # $p < 0.05$ compared to high passage MIN6 cells at 1 mM glucose, @ $p < 0.05$ low passage vs. high passage MIN6 cells at their respective glucose concentrations.

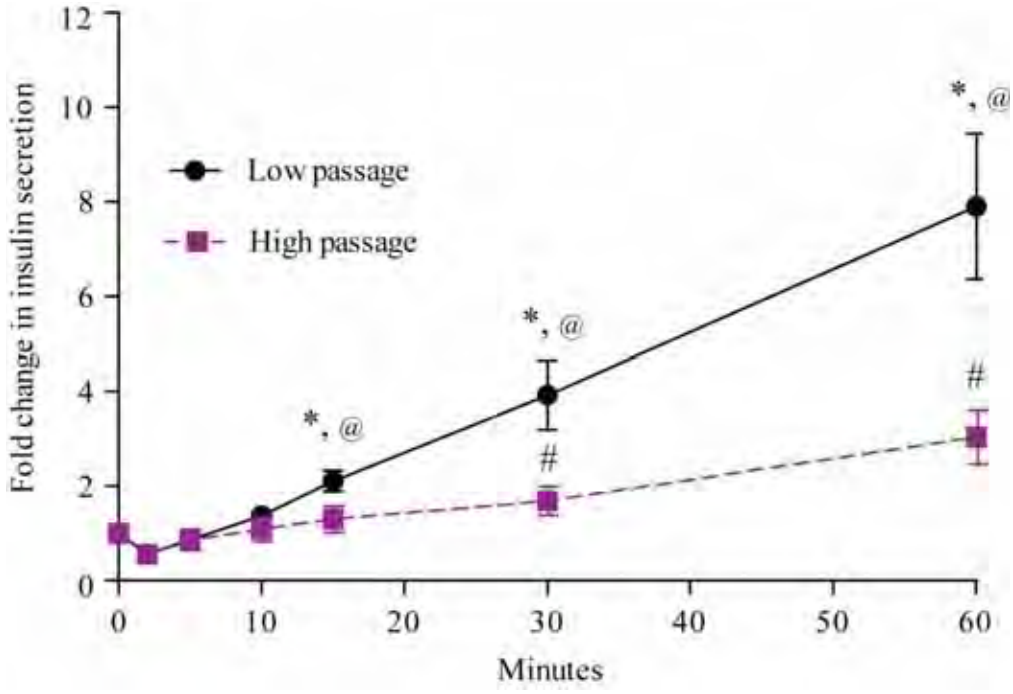

Figure 3.2 GSIS time course in low and high passage MIN6 cells. Low passage MIN6 cells had robust first and second phase insulin secretion. High passage MIN6 cells lacked first phase but retained some second phase insulin secretion however this was significantly less that than that seen in low passage MIN6 cells. * $p < 0.05$ compared to low passage MIN6 cells at time 0, # $p < 0.05$ compared to high passage MIN6 cells at time 0, @ $p < 0.05$ low passage vs. high passage MIN6 cells at their respective time points.

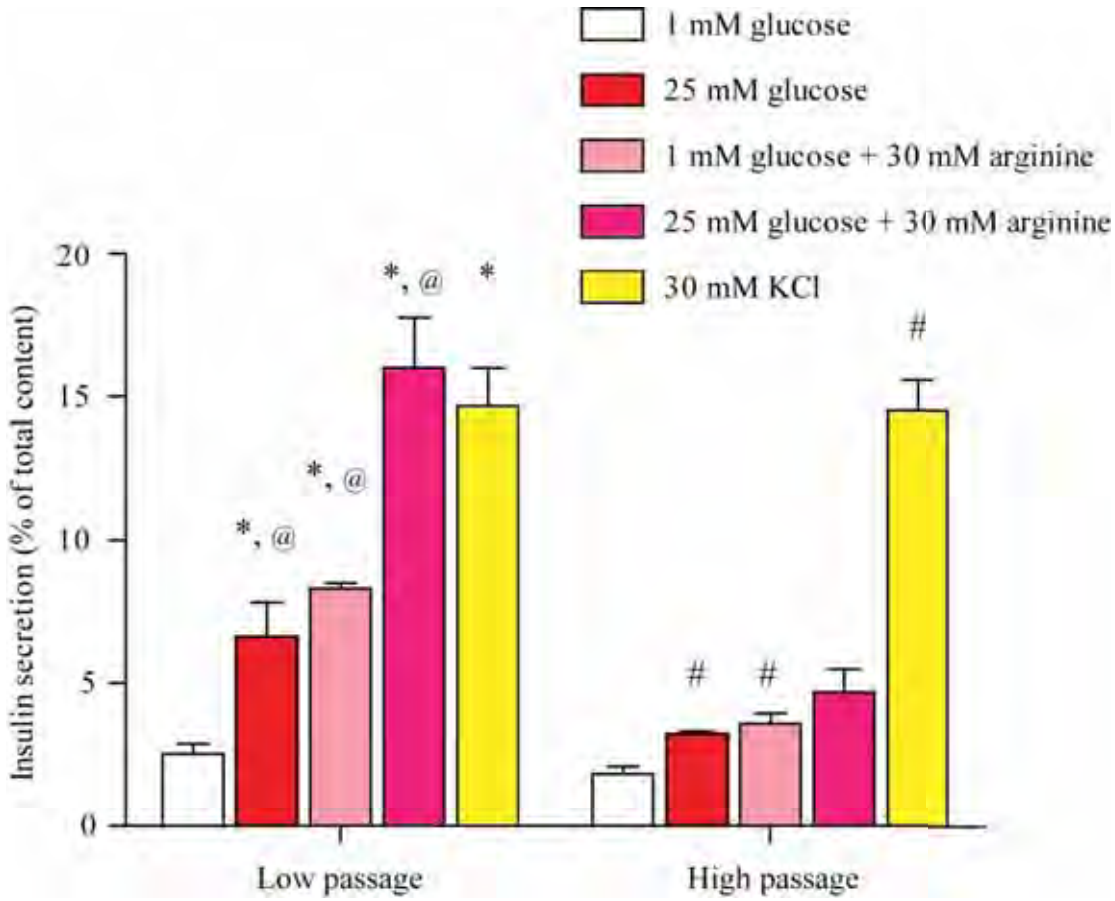

Figure 3.3 Insulin secretion in low and high passage MIN6 cells with glucose and other secretagogues. MIN6 cells were stimulated for 30 minutes in the presence of glucose, 30 mM arginine, and 30 mM KCl. Insulin secretion was augmented in the presence of 30 mM arginine and KCl. Augmentation by 30 mM arginine in high passage MIN6 cells was significantly reduced. Stimulation with 30 mM KCl was not significantly different in low and high passage MIN6 cells. * $p < 0.05$ compared to low passage MIN6 cells at 1 mM glucose, # $p < 0.05$ compared to high passage MIN6 cells at 1 mM glucose, @ $p < 0.05$ low passage vs. high passage MIN6 cells.

3.3.2 Intracellular ATP content in low and high passage MIN6 cells

Low passage MIN6 cells responded to a stimulation of 25 mM glucose with increased ATP content, resulting in significant increases over basal levels from 10 minutes onwards ($p < 0.05$, Figure 3.4). This was not observed in high passage MIN6 cells, with the only significant deviations at 5 and 15 minutes after stimulation (Figure 3.4). Surprisingly, there was a significant decrease in ATP content at 5 minutes after stimulation in high passage MIN6 cells, which was not seen in the low passage cells ($p < 0.05$). The top panel in Figure 3.4 shows the intracellular ATP content expressed as a fold change compared with their respective basal levels. The lower panel in Figure 3.4 shows the same intracellular ATP content data expressed as the raw numbers. Low passage MIN6 cells had markedly higher ATP content, at least 5-fold more, at all time points compared to high passage MIN6 cells ($p < 0.01$).

3.3.3 Morphology and cell cycle progression

Morphologically, low passage MIN6 cells generally appeared round whereas the high passage MIN6 cells had a more irregular shape with pointed protrusions (Figure 3.5). To determine whether high-passage cells might be consuming ATP due to increased cell proliferation, flow cytometry analysis of BrdU incorporation and 7AAD staining in was performed. High passage MIN6 cells had a much greater percentage of cells in the S phase of DNA replication (11.9 % versus 5.4 %, $p < 0.001$, Figure 3.6), which suggests that high passage cells were not utilising ATP by more rapid cell cycling.

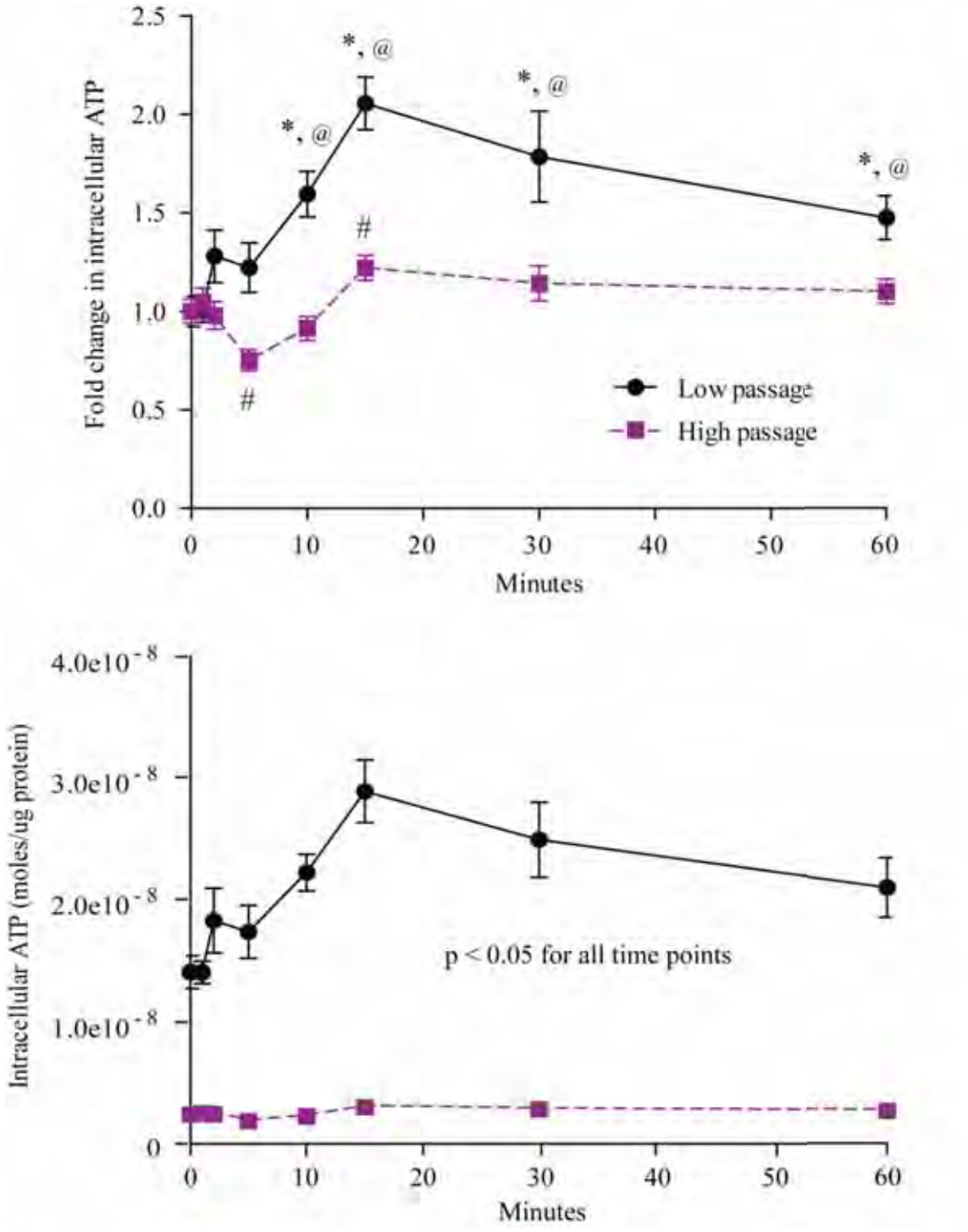

Figure 3.4 Intracellular ATP content time course in low and high passage MIN6 cells. The top figure represents the intracellular ATP content as a fold change compared to their respective basal levels. Low passage cells responded well with glucose stimulation but this was not observed in high passage MIN6 cells. The lower figure represents the data as raw ATP values. High passage MIN6 cells had significantly lower intracellular ATP content at all time points. * $p < 0.05$ compared to low passage MIN6 cells at time 0, # $p < 0.05$ compared to high passage MIN6 cells at time 0, @ $p < 0.05$ low passage vs. high passage MIN6 cells at their respective time points.

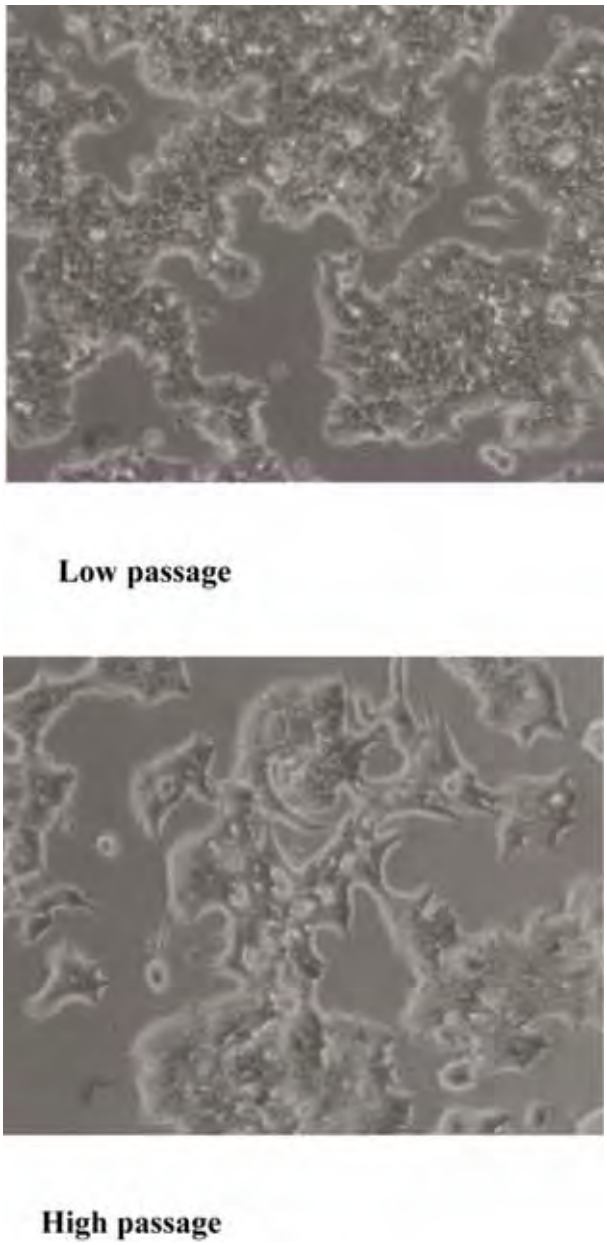

Figure 3.5 Light microscope images of low and high passage MIN6 cells. The top image shows low passage MIN6 cells and the lower figure shows high passage MIN6 cells.

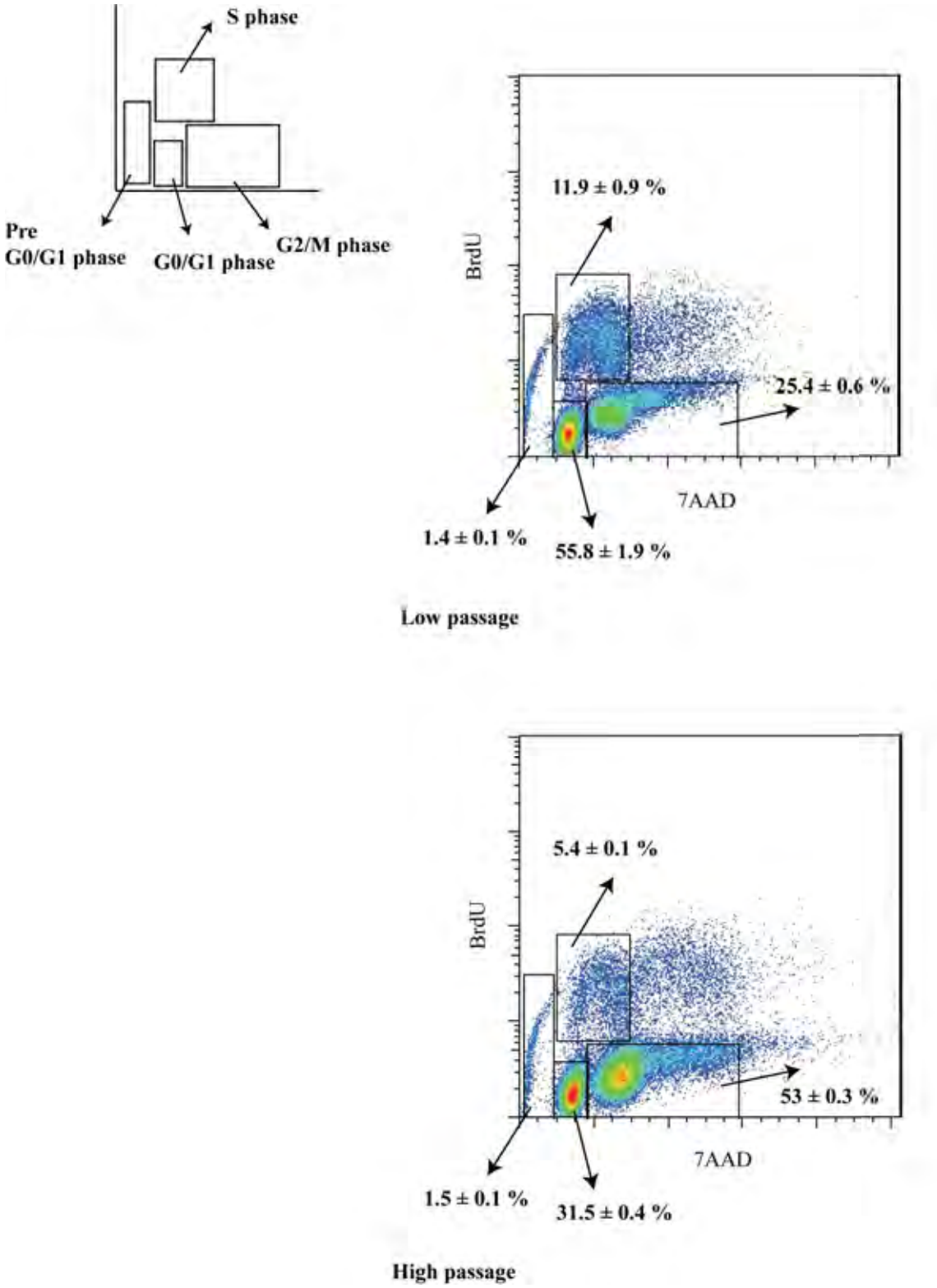

Figure 3.6 Analysis of cell cycling in low and high passage MIN6 cells. MIN6 cells were stained with BrdU and 7AAD. Cells were sorted by FACS and analysed using FlowJo (Tree Star).

3.3.4 Metabolic analysis of low and high passage MIN6 cells

3.3.4.1 Oxygen consumption

Oxygen consumption was measured in low and high passage MIN6 cells to determine if any mitochondrial dysfunction was present. There were no differences observed in the basal rate, nor were there any differences in consumption in the presence of the mitochondrial uncoupler FCCP (Figure 3.7).

3.3.4.2 Glucose uptake

The top panel in Figure 3.8 shows an estimation of the total glucose uptake in the cells represented as the fold change compared to basal levels in low passage MIN6 cells (i.e. including calculated uptake of total glucose including 2-deoxy-[1,2-^{3}H]-glucose). Both low and high passage cells had a significant increase in calculated total glucose (radioactive plus non radioactive) uptake from 1 to 25 mM glucose. This was ~50 % greater in low passage MIN6 cells, however this was not significant (Figure 3.8 top panel). The lower panel in Figure 3.8 shows the fold change in total glucose uptake compared to their respective basal levels. Low passage MIN6 cells had ~38-fold increase from basal while high passage MIN6 cells only had ~7-fold increase from basal (Figure 3.8 lower panel). Interestingly, high passage cells had approximately 3-fold higher glucose uptake at basal glucose versus low passage cells ($p < 0.01$) but decreased uptake at 25 mM glucose suggesting that their basal uptake was at a greater proportion of maximal capacity (Figure 3.8).

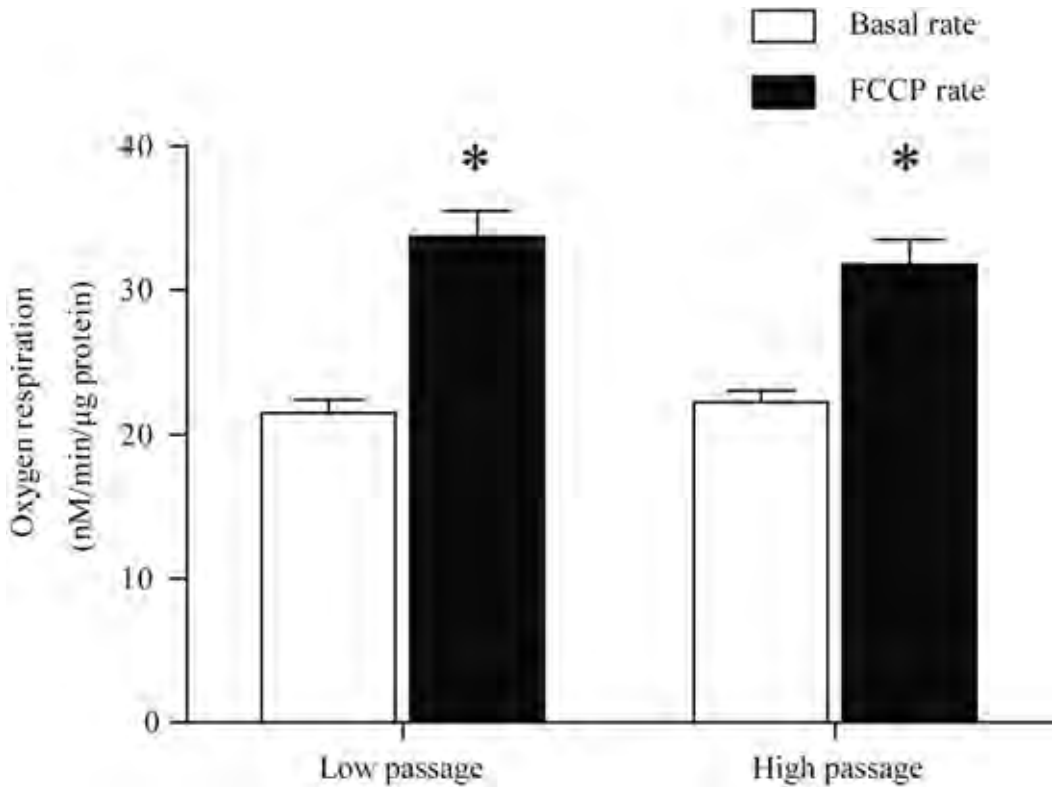

Figure 3.7 Oxygen consumption in low and high passage MIN6 cells. Oxygen consumption was measured in low and high passage MIN6 cells basally and in the presence of the mitochondrial uncoupler FCCP.

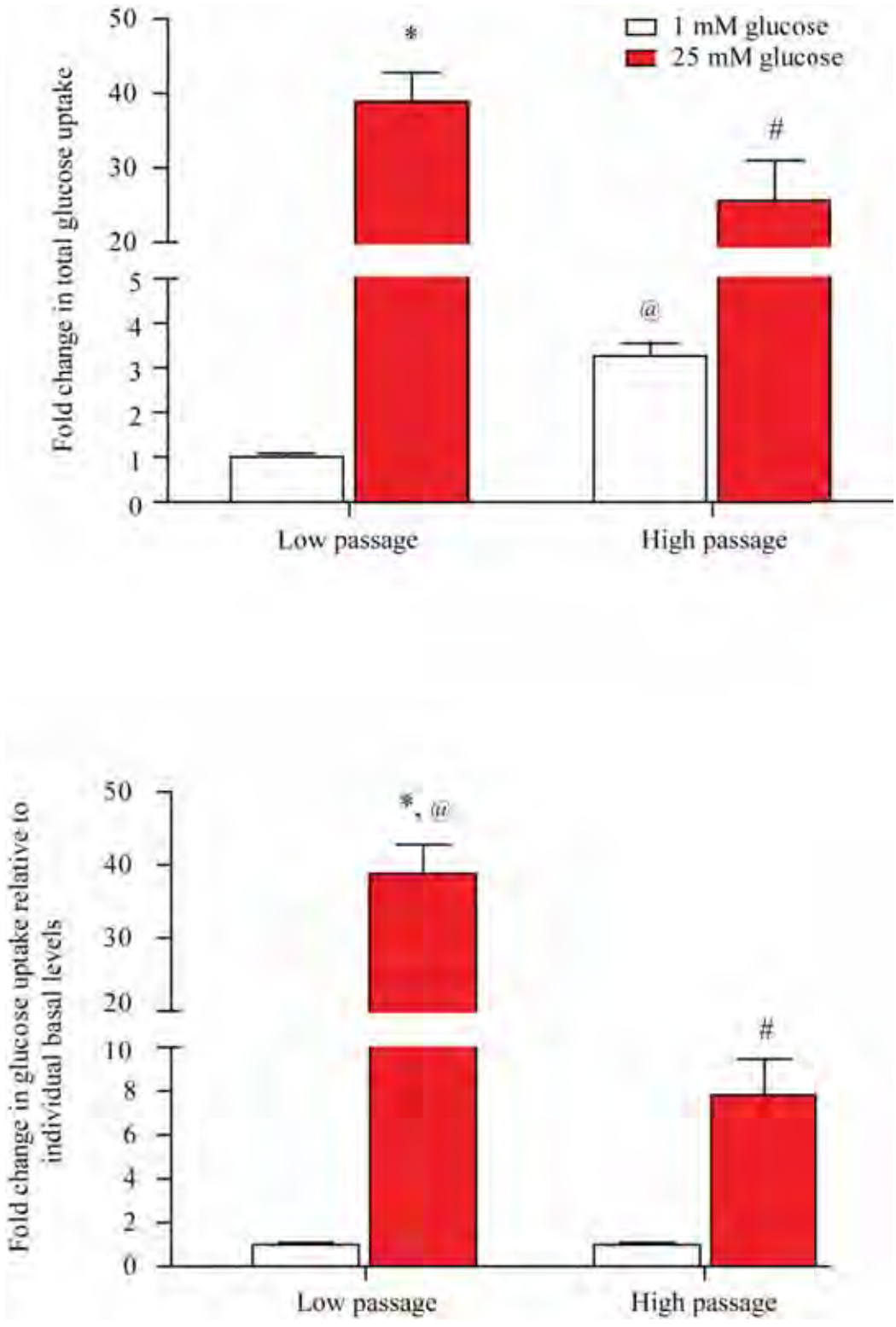

Figure 3.8 Glucose uptake in low and high passage MIN6 cells. The top figure shows the total glucose uptake as a fold change compared to basal levels in low passage MIN6 cells. The lower figure shows the total glucose uptake compared to their respective basal levels. * $p < 0.05$ compared to low passage MIN6 cells at 1 mM glucose, # $p < 0.05$ compared to high passage MIN6 cells at 1 mM glucose, @ $p < 0.05$ low passage vs. high passage MIN6 cells.

3.3.5 Glucose oxidation

Four glucose concentrations were tested; 1, 5, 11, and 25 mM. Low passage cells exhibited a dose dependent increase in glucose oxidation with an approximate 28-fold increase from 1 to 25 mM glucose ($p < 0.001$, Figure 3.9 top panel). Consistent with their increased basal glucose uptake, high passage cells had an 8-fold higher basal glucose oxidation compared to low passage cells ($p < 0.001$). High passage MIN6 cells had only a 1.8-fold increase in glucose oxidation at 25 mM glucose (Figure 3.9 lower panel). This resulted in 50 % lower absolute glucose oxidation at 25 mM glucose in high versus low passage MIN6 cells (Figure 3.9 top panel).

3.3.6 Lipid oxidation

Lipid oxidation provides an important source of energy to β-cells. In low passage cells, lipid oxidation was high at 1 mM glucose and as expected [271], was markedly reduced at 25 mM glucose (~6-fold, $p < 0.0001$, Figure 3.10). In contrast, basal lipid oxidation in high passage MIN6 cells was low ($p < 0.0001$ vs. low passage MIN6 cells at basal) and there was no significant change at 25 mM glucose (Figure 3.10).

3.3.6.1 GSIS with bromopalmitate treatment

To determine whether the loss of basal lipid oxidation was important for the phenotype of high passage MIN6 cells, bromopalmitate was used to inhibit lipid oxidation. High concentrations (0.5 μM) of bromopalmitate caused rapid cell death within 2 hours, indicating the importance of lipid oxidation (data not shown). There is an indication of increased insulin secretion with the addition of 0.0625 μM bromopalmitate for 2 hours (Figure 3.11 top panel), however this was possibly influenced by the high amount of cell death caused by the bromopalmitate. The same data is expressed in raw numbers (without accounting for total insulin levels) and assuming the same cell density, the

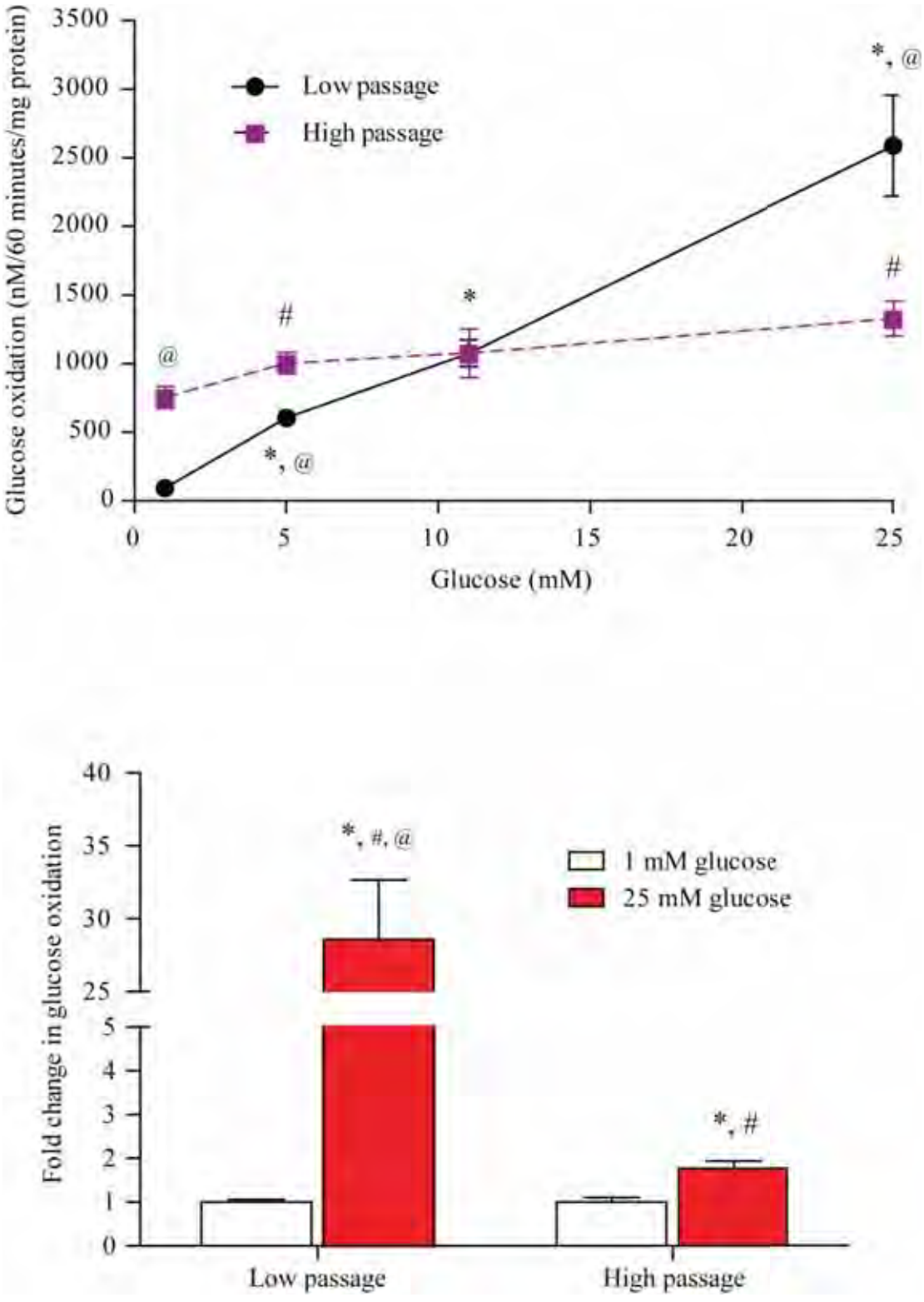

Figure 3.9 Glucose oxidation in low and high passage MIN6 cells. The top figure shows the glucose oxidation for various glucose concentrations in low and high passage MIN6 cells. The lower figure shows the fold change in glucose oxidation as a fold change compared to their respective basal levels. * $p < 0.05$ compared to low passage MIN6 cells at 1 mM glucose, # $p < 0.05$ compared to high passage MIN6 cells at 1 mM glucose, @ $p < 0.05$ low passage vs. high passage MIN6 cells.

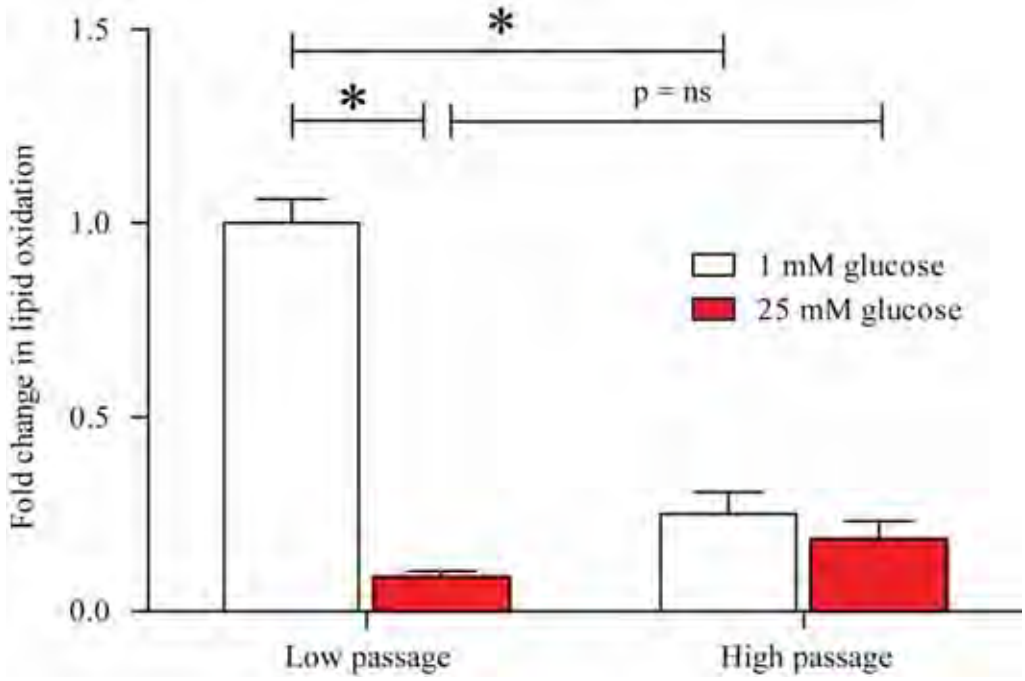

Figure 3.10 Lipid oxidation in low and high passage MIN6 cells. Lipid oxidation was measured and expressed as the fold change compared to basal levels in low and high passage MIN6 cells. * $p < 0.05$.

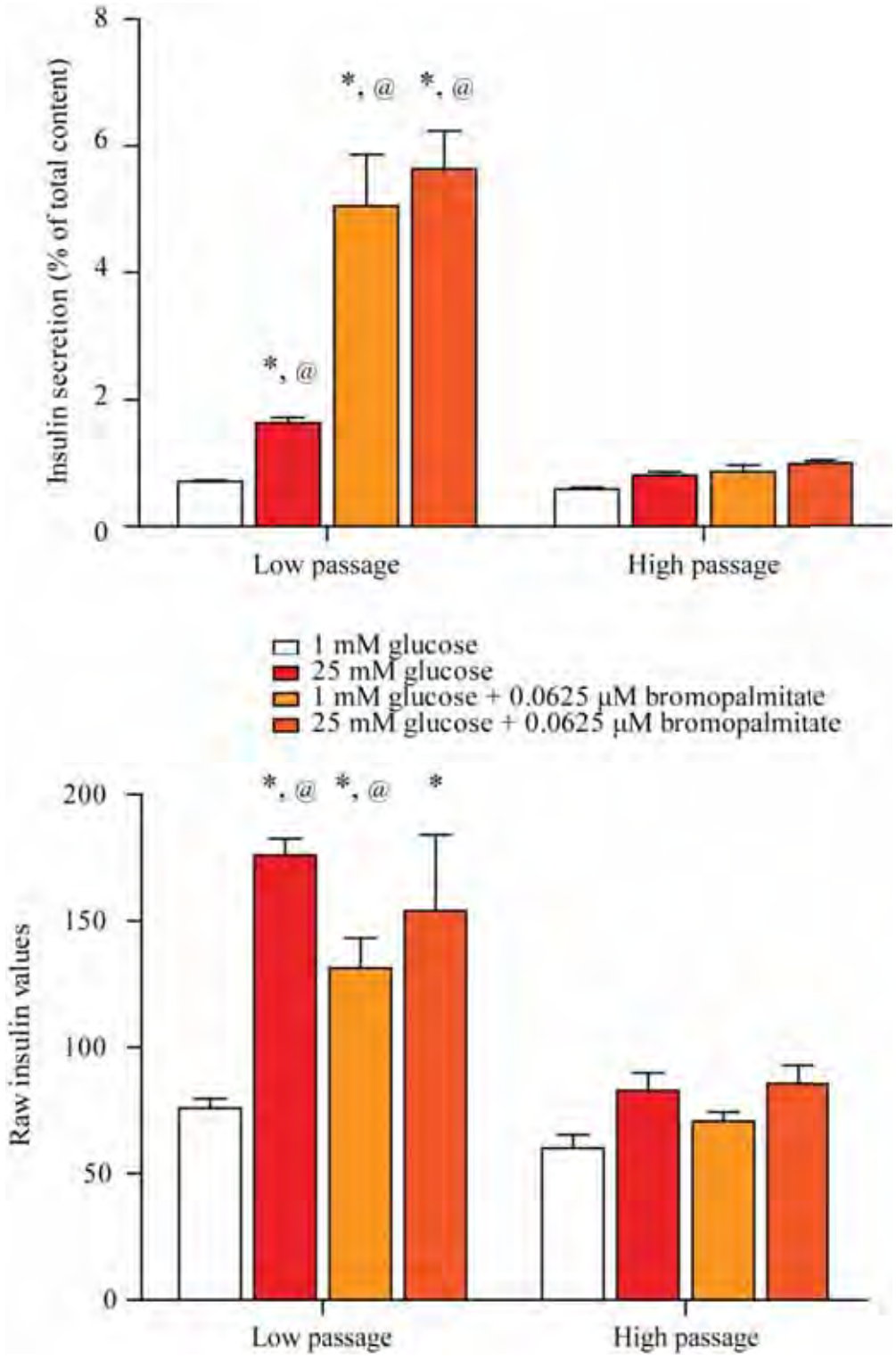

Figure 3.11 GSIS after treatment with bromopalmitate. Low and high passage MIN6 cells were treated with 0.0625 μM bromopalmitate for 2 hrs before insulin secretion samples were taken. The top figure shows insulin secretion as the percentage of total insulin and the lower figure shows the raw insulin values without correcting for total insulin content. * $p < 0.05$ compared to low passage MIN6 cells at 1 mM glucose, @ $p < 0.05$ low passage vs. high passage MIN6 cells.

inhibition of lipid oxidation with 0.0625 μM bromopalmitate had no significant impact in both low and high passage MIN6 cells (Figure 3.11 lower panel).

3.3.7 Lactate

To exclude the possibility that glucose oxidation was decreased due to shunting into lactate synthesis rather than metabolism of glucose, we measured expression of *Ldh-A* and lactate. No significant differences in lactate were observed between 1 and 25 mM glucose in low passage MIN6 cells (Figure 3.12). High passage MIN6 cells had a non-significant decrease in lactate at 1 mM glucose and a significant increase at 25 mM glucose (Figure 3.12) but this was still lower than the levels in low passage cells. High passage MIN6 cells had a 2-fold increase in *Ldh-A* expression ($p<0.00001$, Figure 3.13).

3.3.8 Gene expression changes in low and high passage MIN6 cells

High passage MIN6 cells had reduced expression of mRNAs encoding the glucose transporter *Glut1*, *Hnf4α,* and *Pfk* ($p < 0.01$, Figure 3.13). High passage MIN6 cells had increased expressions of *Hk1* and *Hk2*, with *HK1* increased approximately 60-fold ($p < 0.01$, Figure 3.14). The key glycolytic gene *Gck* (also known as *Hk IV*) was decreased in high passage MIN6 cells ($p < 0.01$, Figure 3.14). Analysis of lipid handling genes revealed that high passage MIN6 cells had reduced expression of the important lipid synthesis transcription factor *sterol regulatory element binding protein 1C* (*Srebp1c*, $p < 0.0001$, Figure 3.15) and decreased cholesterol synthesising genes *HMG coA reductase* and *HMG coA synthase* mRNAs. High passage MIN6 cells had lower expression of *L-β-hyroxyacyl coA dehydrogenase (Hadh)* and higher expression of *acyl-coA dehydrogenase medium chain (Acadm)*, *enoyl coA hydrotase (Ehhadh),* and *acetyl-coA acyltransferase 1a (Acaa1a)* (Figure 3.16).

The mitochondrial uncoupling gene, *Ucp2*, was decreased by 35 % in high passage MIN6 cells ($p < 0.0001$, Figure 3.17). Only very low levels of *Ucp1* mRNA were present in both low and high passage MIN6 cells and these did not differ (data not shown). The sirtuin *Sirt3* has been reported in other tissues to regulate *Nampt* and lipid handling. Expression of both *Nampt* and *Sirt3* was significantly decreased in high passage MIN6 cells (Figure 3.17). The mRNA expression of the pancreatic transcription factor *pancreatic duodenal homeobox gene-1* (*Pdx1*) and *insulin 2* were examined. High passage MIN6 cells had an approximate 50 % increase in *Pdx1* expression ($p<0.00001$) and an approximate 60 % decrease in *insulin 2* expression ($p<0.00001$, Figure 3.18).

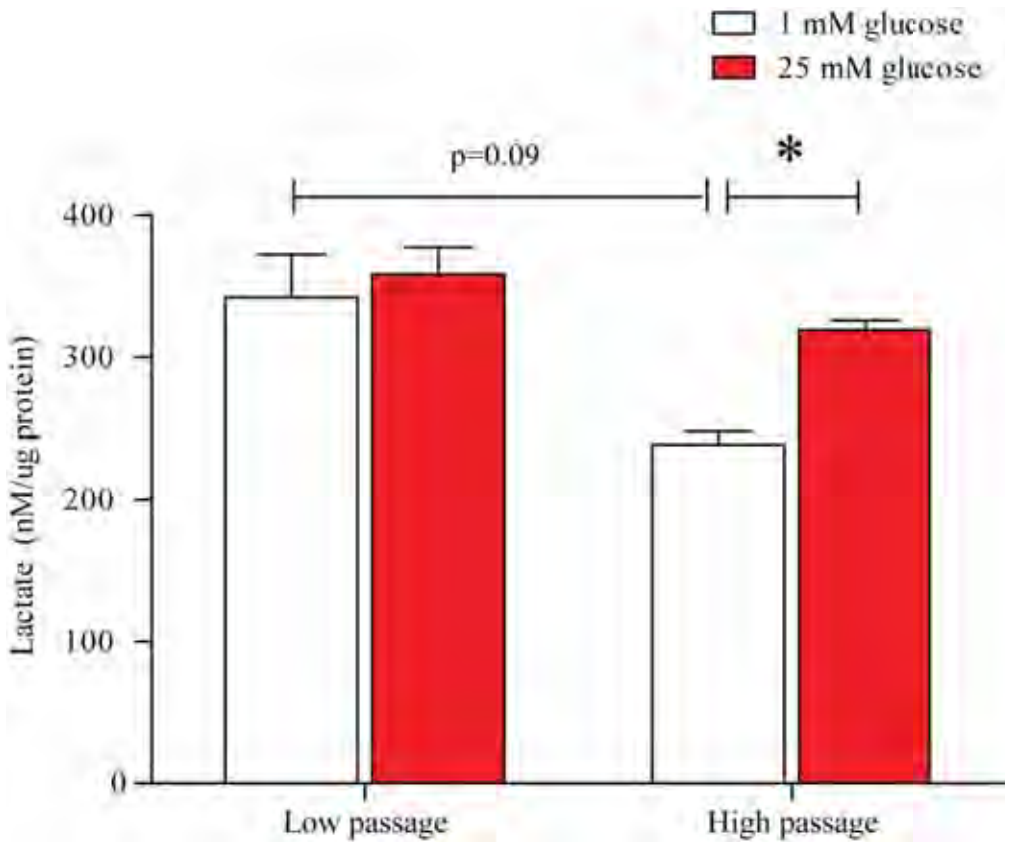

Figure 3.12 Lactate in low and high passage MIN6 cells. Lactate was measured in low and high passage MIN6 cells. * $p < 0.05$.

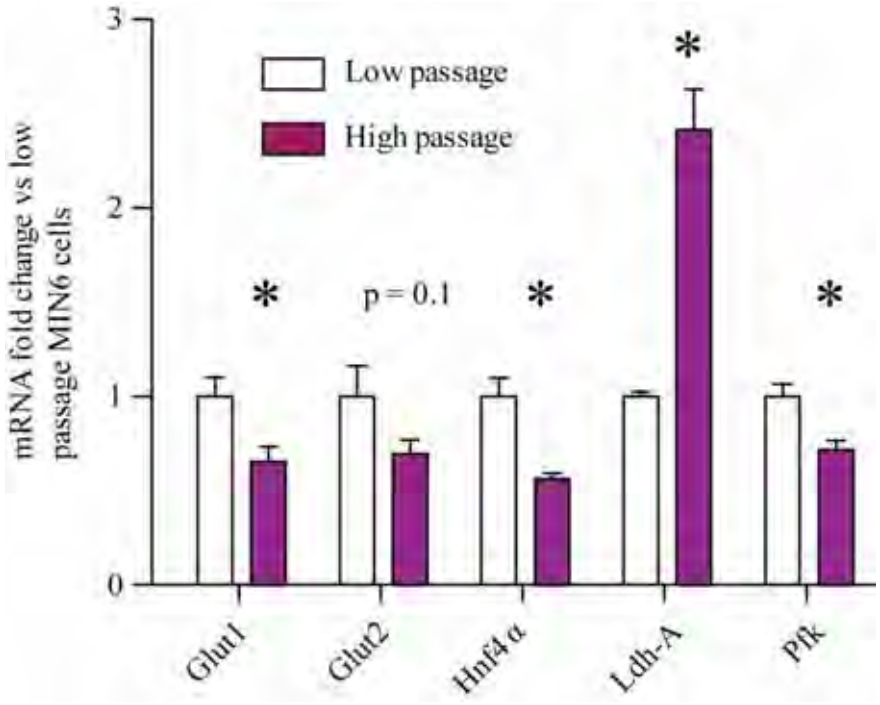

Figure 3.13 RT-PCR in low and high passage MIN6 cells. Some glycolytic genes and also *Hnf4α* gene expression levels were determined in low and high passage MIN6 cells. * $p < 0.05$.

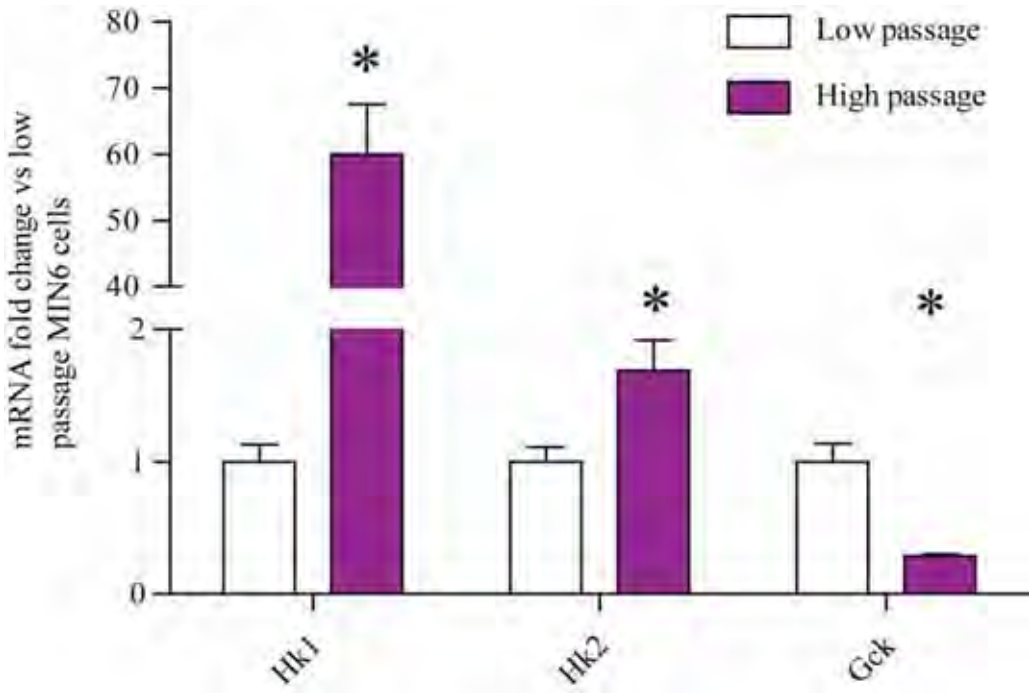

Figure 3.14 RT-PCR in low and high passage MIN6 cells. The glycolytic genes *Hk1*, *Hk2*, and *Gck* were determined in low and high passage MIN6 cells. * $p < 0.05$.

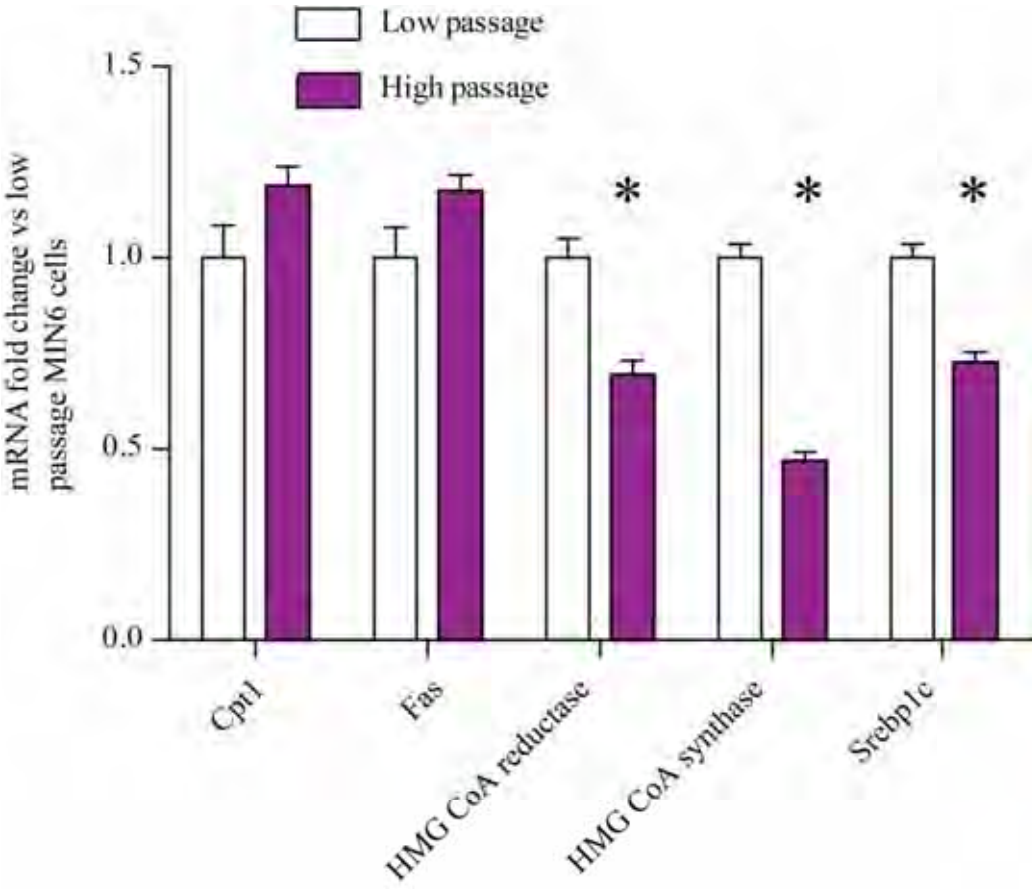

Figure 3.15 RT-PCR in low and high passage MIN6 cells. Some lipid handling gene expression levels were determined in low and high passage MIN6 cells. * $p < 0.05$.

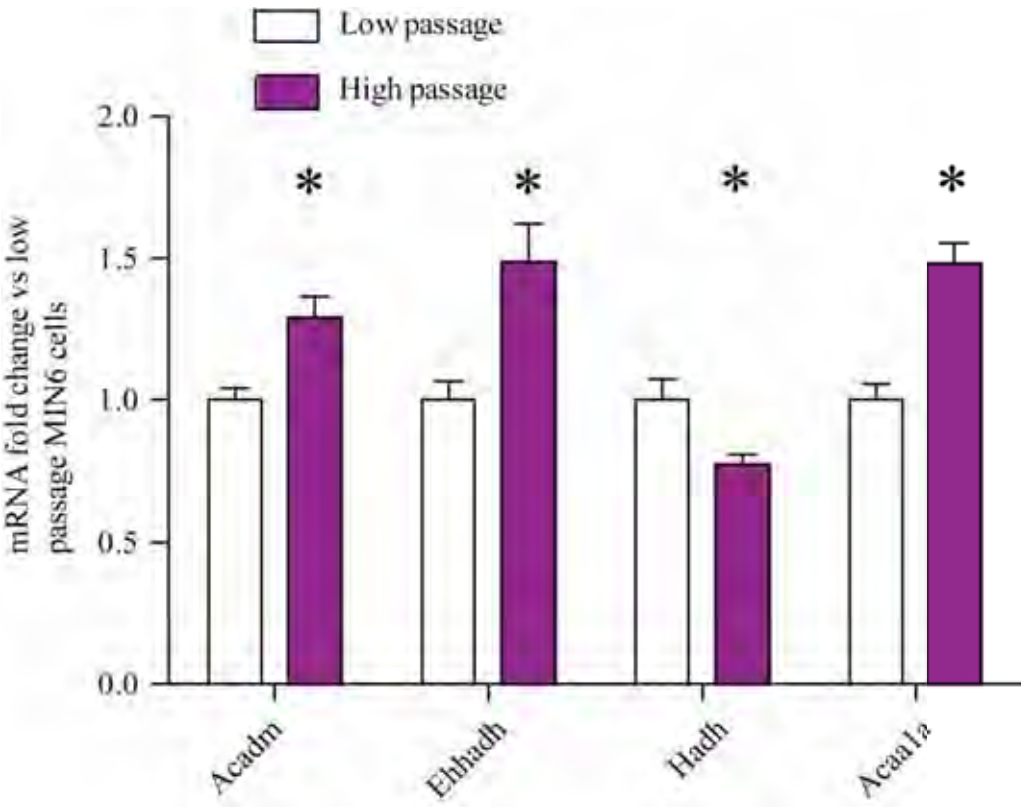

Figure 3.16 RT-PCR in low and high passage MIN6 cells. Some lipid oxidation gene expression levels were determined in low and high passage MIN6 cells. * $p < 0.05$.

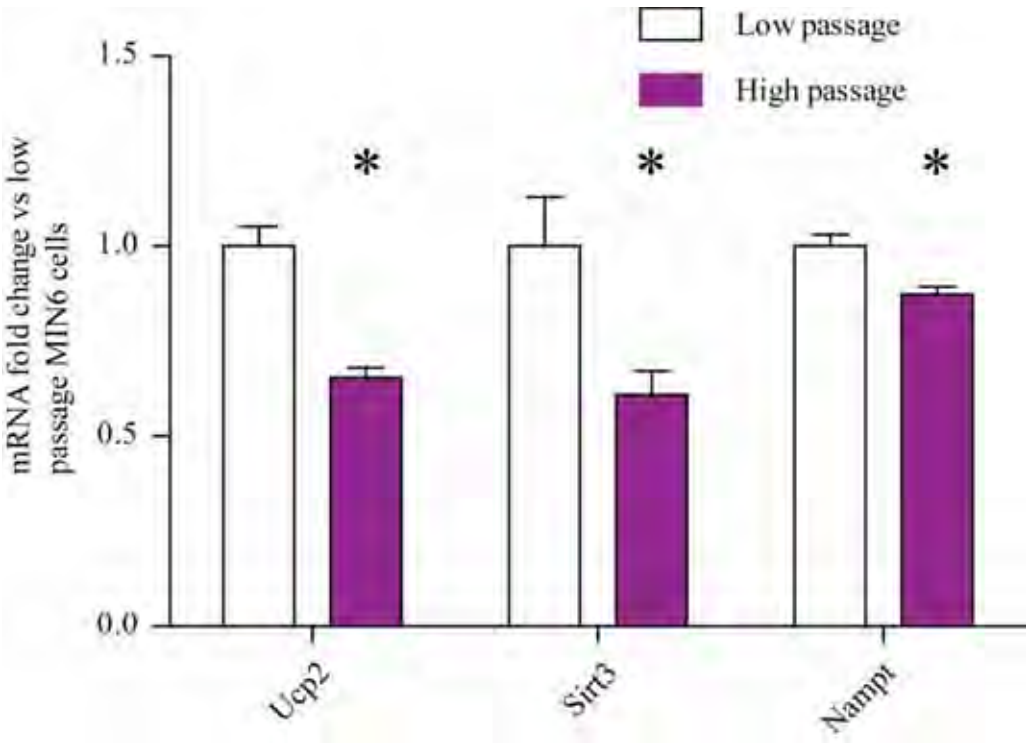

Figure 3.17 RT-PCR in low and high passage MIN6 cells. *Ucp2*, *Sirt3*, and *Nampt* gene expression levels were determined in low and high passage MIN6 cells. * $p < 0.05$.

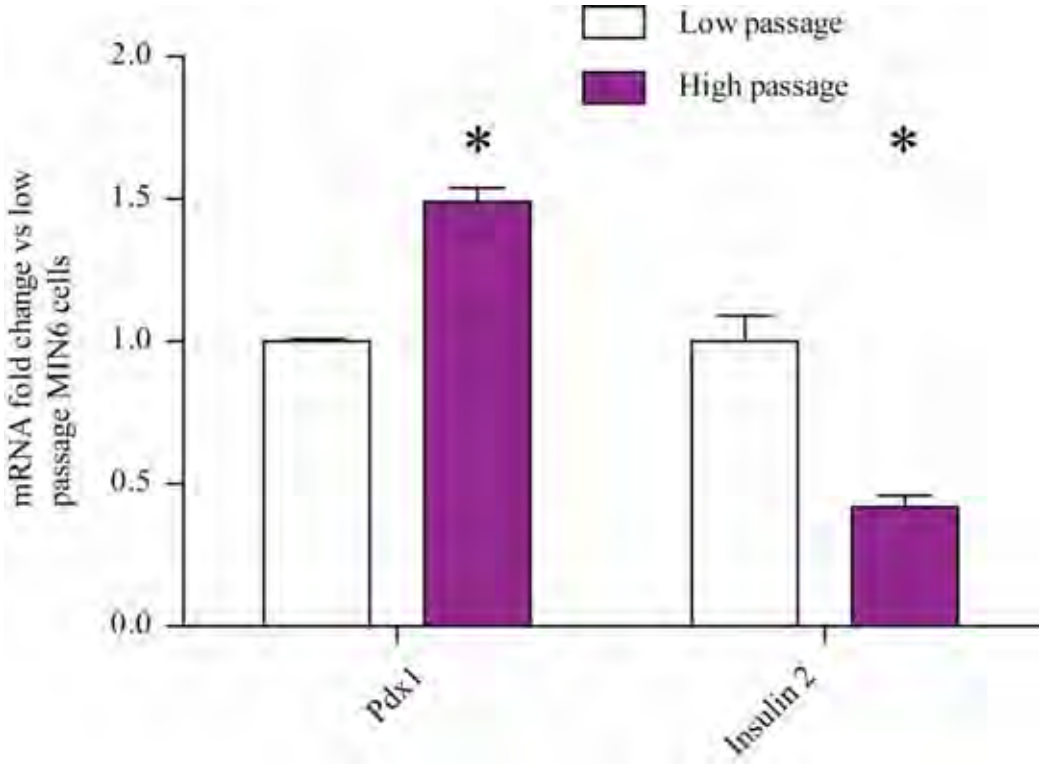

Figure 3.18 RT-PCR in low and high passage MIN6 cells. *Pdx1* and *Insulin 2* gene expression levels were determined in low and high passage MIN6 cells. * $p < 0.05$.

3.4 Discussion

Subjects with T2D lose first phase insulin secretion early in the natural history of disease progression [52, 272-274]. In this study, we used the MIN6 cell line to examine defects in first phase insulin secretion. With high passage these cells lose first phase and partially retain second phase GSIS. Previous studies in low and high passage MIN6 cells have shown both expression and protein level changes [266-268]; however this is the first report to profile metabolic changes. HIT-T15 cells, another clonal β-cell type, are also known to lose both first and second phase GSIS with increased passage. This was attributed to decreased insulin gene expression and insulin content and was suggested to be related to constant exposure to 11mM glucose [275]. Our high passage MIN6 cells displayed similar characteristics, with reduced *insulin 2* mRNA expression. The morphology and insulin secretion profile of our high passage MIN6 cells correspond to the glucose non-responsive MIN6 cells previously described by Lilla et al [269]. And while our gene profiling was not as exhaustive, we also found a non-significant change in *Glut2* mRNA expression.

High passage MIN6 cells responded well to KCl, a non-glucose insulin secretagogue that works by closing the K_{ATP} channels, downstream of glucose metabolism. Normal KCl-stimulated insulin secretion in high passage cells clearly indicated that the defects are not in insulin synthesis or secretion itself but lie upstream. L-arginine stimulation of insulin secretion was retained in high passage MIN6 cells, but to a much lesser extent compared to that observed in low passage MIN6 cells. L-arginine stimulates insulin by inducing Ca^{2+} release from mitochondria via the actions of nitrogen oxides in the presence of glucose [276, 277]. These changes again suggest that the defects lie upstream of the K_{ATP} channels.

The lack of an increase in early insulin secretion response in high passage MIN6 cells was consistent with the failure of increase in intracellular ATP concentration when

stimulated with glucose. An increase in ATP:ADP ratio is crucial in the β-cell as this precedes a cascade of steps necessary for insulin secretion [85, 132, 134]. High passage MIN6 cells had significantly decreased intracellular ATP at 5 minutes after glucose stimulation but the mechanism or the significance of this is unknown. It is possible that ATP consuming events such as glycolysis could be increased shortly after glucose stimulation without subsequent ATP generation but this needs to be verified. However, impaired intracellular ATP content was ultimately a result of impaired basal lipid oxidation and decreased glucose uptake and glucose oxidation in response to changes in glucose exposure, as no differences in mitochondrial oxygen respiration was observed. Glucose uptake was significantly impaired and this was associated with a significant decrease in *Glut1* and a trend to decrease in *Glut2* in high passage MIN6 cells. GLUT2 is the main glucose sensor in the β-cell due to its high K_m [170]. There was also a reduction in other important glycolytic genes including the rate limiting glycolytic enzymes *Gck* and *Pfk*, both of which are important in the provision of substrates to the TCA cycle and oxidative phosphorylation. The low K_m hexokinases *Hk1* and *2* were significantly increased in high passage MIN6 cells, which could be a possible attempt to increase glycolysis and substrates for mitochondrial oxidative phosphorylation. A previous report has shown that isolated rat islets overexpressing *Hk1* have increased insulin secretion and glucose oxidation at basal glucose levels, much like that seen in high passage MIN6 cells [278].

Mitochondrial oxidative phosphorylation plays an important role for insulin secretion, as it provides much of the needed ATP to change the ATP:ADP ratio [142-144]. The question of the relative contribution of mitochondrial oxidative phosphorylation versus other pathways in physiological insulin secretion remains controversial [149, 151-153]. Regardless, in order for oxidative phosphorylation to be able to generate ATP, substrates must be provided to it by the glycolytic pathway and

the TCA cycle. This flow of substrates could be inhibited by high *Ldh-A* expression. LDH-A converts pyruvate to lactate and NAD^+ and thus decreases pyruvate. Over-expression of *Ldh-A* in MIN6 cells has been shown to attenuate GSIS [279]. However, lactate in high passage MIN6 cells was actually decreased, suggesting that this was not a mechanistic change. There was also no change in oxygen respiration rates as measured by a Clark-type oxygen electrode.

Some lipid synthesis genes were also down-regulated in high passage MIN6 cells, including the important transcription factor *Srebp1c*. Lipids, as well as glucose, are a major source of ATP. Per Mole of substrate, lipid yields more ATP than glucose with up to 136 molecules of ATP generated per palmitate molecule versus 30-36 ATP per glucose molecule. Rough calculations estimating the amount of ATP able to be produced from glucose and lipid oxidation indicate that high passage MIN6 cells produce more intracellular ATP compared to low passage MIN6 cells at 1mM glucose. This suggests that high passage MIN6 cells are either utilising much more ATP at 1mM glucose or they are wasting energy. High passage cells MIN6 cells, morphologically different to low passage MIN6 cells, were not utilising extra energy in increased cell proliferation as they had a slower rate of cell division, as evidenced by FACS analysis of the BrdU uptake.

The uncoupling proteins function by dissipating the energy from glucose/lipid oxidation as heat rather than flowing through the electron transport chain to produce ATP [208]. The uncoupling protein UCP1 is the classic and most well known UCP out of five (UCP1 – UCP5) but is predominantly expressed in brown adipose tissue [209, 210]. There were very low levels of *Ucp1* expression in both low and high passage MIN6 cells and these were not significantly different. We measured *Ucp2* expression as this has been proposed to be a major factor in obesity, β-cell dysfunction, and T2D, negatively regulating insulin secretion [211]. Recent findings have proposed that UCP2

does not act as an uncoupler and does not contribute to adaptive thermogenesis [210, 280-282]. In fact, a previous study has shown that a decrease in intracellular ATP content down-regulated *Ucp2* expression in mouse hepatocytes [283] and mice with homozygous knockout of *Ucp2* have impaired β-cell function, possibly due to increased oxidative stress [213]. Interestingly, high passage MIN6 cells had a 35 % lower expression in *Ucp2*. The decreased *Ucp2* in high passage MIN6 cells may be secondary to the markedly decreased ATP content.

Recent findings have indicated the enzyme NAMPT and NAD biosynthesis in insulin secretion and metabolism [200, 201]. The enzyme NAMPT is the rate limiting step in NAD biosynthesis and mice with a heterozygous deletion of *Nampt* have been shown to have impaired glucose tolerance and isolated islets have reduced GSIS [201]. There is also reduced expression of *Sirt3* in streptozotocin induced diabetic mice [207]. The sirtuins are a family of deactetylases and mono-ADP-ribosyltransferases, of which there are seven in mammals, that use NAD as a substrate [202]. It has been previously reported that Sirt1 regulates insulin secretion by repressing *Ucp2* in β-cells [203] but there are no reports regarding Sirt3 and β-cell function. High passage MIN6 cells had significantly reduced expression of both *Nampt* and *Sirt3*. Sirt3 regulates mitochondrial fatty acid oxidation in other tissues and *Sirt3*$^{-/-}$ mice have reduced ATP levels in various tissues [284, 285]. High passage MIN6 cells had significantly reduced *Sirt3* expression and reduced lipid oxidation, suggesting that decreased Sirt3 may be driving this.

As lipid oxidation at 1mM glucose failed to provide the high passage MIN6 cells with ATP, we believe that they attempted to compensate for this by increasing glucose uptake and oxidation in the basal state. To determine the importance of basal lipid oxidation, it was blocked with the non-metabolisable lipid bromopalmitate, but that caused cell death and further reductions in insulin secretion.

This data show that high passage MIN6 cells have a very different metabolic profile compared to low passage MIN6 cells. Clearly, some of these metabolic changes are causative while some are compensatory in response to a failure in increasing intracellular ATP. It is interesting to note that many of these changes also occur in patients with type 2 diabetes - impaired insulin response to L-arginine [286], decreased glucose oxidation [287], increased whole body glucose uptake at basal glucose levels [287], and decreased *Gck*, *Hnf4α*, and *Pfk*, and increased *Pdx1* expression [74, 172].

3.4.1 Conclusion

In conclusion, the metabolic assays performed in this project have identified the following:

i) High passage MIN6 cells have loss of first phase insulin secretion but retain some second phase secretory capacity. These cells also respond well to the non-glucose secretagogue KCl.

ii) High passage MIN6 cells had markedly reduced intracellular ATP content compared to low passage MIN6 cells and this was not due to increased cell cycling.

iii) High passage MIN6 cells displayed a different metabolic profile to low passage MIN6 cells. Glucose uptake, glucose oxidation, and lipid oxidation were all altered in high passage MIN6 cells, possibly as an adaptive mechanism to increase ATP production for insulin secretion.

iv) Gene expression changes were observed in high passage MIN6 cells. These included some key glycolytic genes including *Hk1*, *Gck* and *Glut1*. There were also reduced expression in some lipid handling and lipid oxidation genes. *Ucp2*, *Nampt*, and *Sirt3* expression were also reduced in high passage MIN6 cells.

We hypothesise that reduced *Sirt3* and *Nampt* expression are a potential mechanism underlying these metabolic changes, contributing to the decreased lipid oxidation and decreased *Glut1* and glycolytic gene expression. This led to impaired glucose uptake and a decrease in glucose oxidation (Figure 3.19).

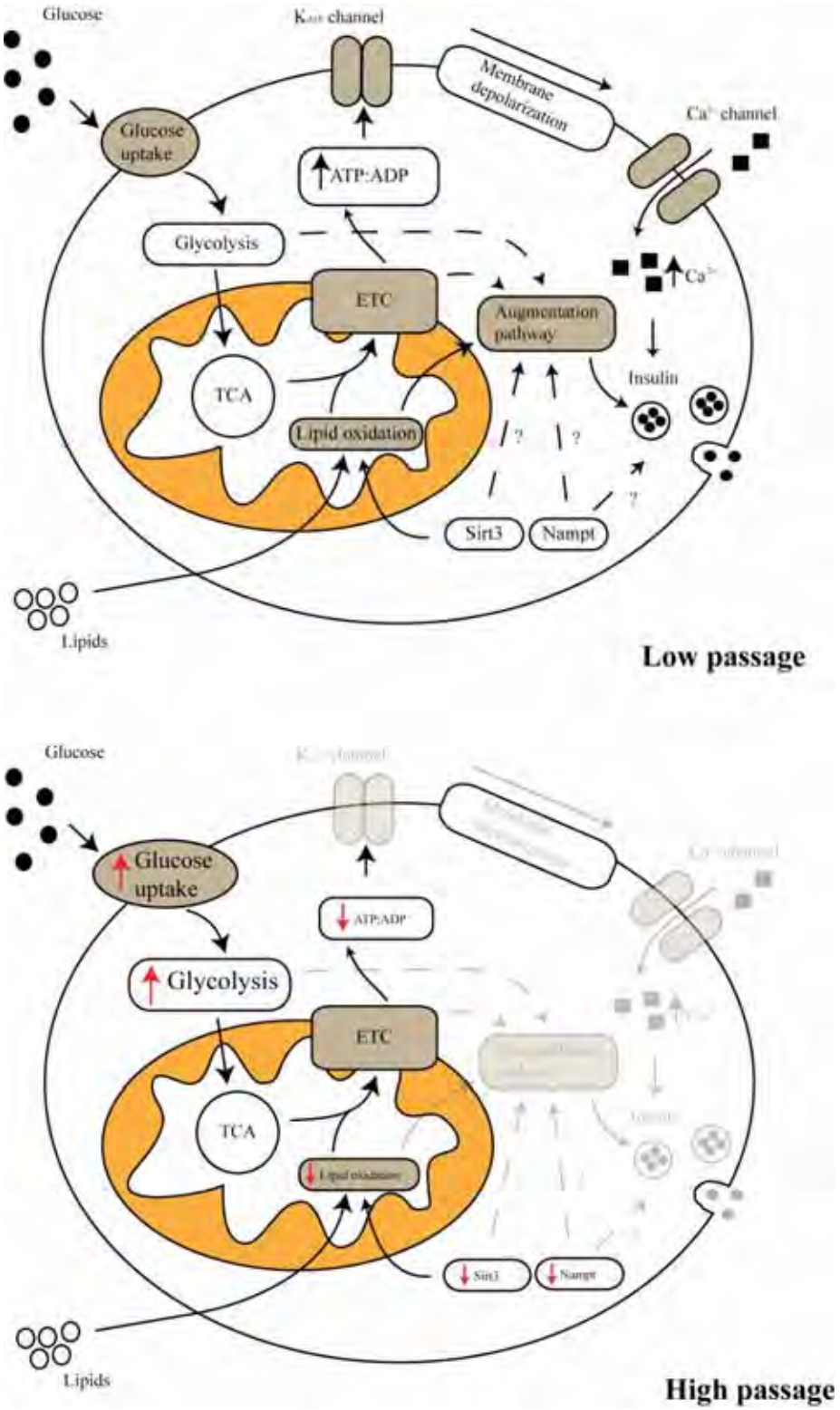

Figure 3.19 Schematic of GSIS in low and high passage MIN6 cells. High passage MIN6 cells have increased glucose uptake, increased glycolysis, decreased ATP generation possibly due to reduced oxidative phosphorylation and decreased lipid oxidation. These all contribute to decreased insulin secretion in high passage MIN6 cells.

Chapter 4

HIF-1α regulation of insulin secretion in mice and MIN6 cells

4.1 Introduction

The transcription factor HIF-1α is important for a range of functions, including cellular responses to hypoxia and other stressors, angiogenesis, and foetal development [227, 288-292]. It has strong anti-apoptotic effects [122, 241, 293-295] and is implicated in the pathogenesis of cardiovascular diseases and some cancers [296-304]. HIF-1α is a member of the bHLH-PAS family [219, 290, 302] and functions as an obligate dimer with other family members, including aryl hydrocarbon receptor (AHR) and ARNT.

Gunton et al. previously reported that ARNT was decreased in islets isolated from patients with T2D and that decreasing ARNT in MIN6 cells or disrupting it in mouse β-cells caused changes in gene expression and GSIS similar to those seen in islets isolated from humans with T2D [172]. A recent study using laser capture microdissection showed no changes in ARNT between control and T2D islets and the author states these discrepancies could be due to the sample preparation process [305]. However, the additional experiments in the form of knockout mice and knockdowns in cell culture performed by Gunton et al. would support the notion that ARNT is indeed important for β-cell function. Recently, Wang et al. reported a loss of ARNT expression in the livers in people with T2D, affecting dysregulation of gluconeogenesis [306]. Though the specific ARNT partner which is important for its actions in β-cells (or liver) is not known, candidates include AhR, HIF-1α, HIF-2α, HIF-3α, and circadian rhythm molecules, e.g., BMAL.

Because of its role in the regulation of glycolysis and other biological processes in other tissues [307, 308], we hypothesised that (a) HIF-1α might be the important partner for ARNT in β-cells, (b) that decreasing HIF-1α would impair β-cell reserve and thus lead to diabetes under conditions of β-cell stress, and (c) that increasing HIF-1α in a nontoxic way would improve β-cell function.

Consistent with its role in regulating a number of important biological processes, HIF-1α protein is tightly regulated [219, 290, 301, 303, 308, 309]. In the basal state, it is hydroxylated on proline residues and becomes competent to associate with VHL protein, leading to ubiquitination and rapid proteolysis, giving a half-life of minutes [303, 310, 311]. Oxygen, iron, and 2-oxoglutarate are required for hydroxylation [238, 312-314]. Thus, hypoxia inhibits degradation, leading to a rapid increase in HIF-1α levels. In addition, HIF-1α protein can be increased by genetic inactivation of VHL or the hydroxylases, treatment with heavy metals such as cobalt chloride, or iron chelation with DFO or DFS [304, 312]. An additional layer of regulation is added by asparaginyl-hydroxylation, which inhibits association with transcriptional cofactors, including p300 [219].

Until recently, it was thought that HIF-1α did not function under normoxic conditions. However, the presence of HIF-1α protein in brain, kidney, liver, embryonic stem cells, trophoblastic cells, and others [226, 227, 288] is now recognised. It is stabilised by inflammation, transforming growth factor, platelet derived growth factor, epidermal growth factor, interleukin-1β [304, 315, 316], and by increased levels of ROS [317-319]. Of potential relevance to β-cells, insulin increases HIF-1α activity in liver, muscle, breast carcinoma, prostate carcinoma, and retinal epithelial-derived cells [320-323]. The PI3K-Akt pathway activation is required for the insulin-induced increase [324].

The role of HIF-1α in islets is not fully understood. Pancreatic islets are normally exposed to relatively low oxygen tension (20 – 37 mmHg) [325, 326] and to locally secreted insulin. These factors suggest a possible role for HIF-1α in islets and the possibility for decreased HIF-1α in the setting of insulin resistance.

4.2 Aims and strategies

We sought to characterise the role of HIF-1α in regards to glucose homeostasis in both an animal and cell culture model. In all models, we aim to conduct experiments to determine any changes in glucose tolerance, insulin secretion, gene expression, and intracellular ATP content. The models that will be used include:

i) Using targeted disruption of *Hif-1α* in β-cells of C57BL/6 mice (referred to herein as β-*Hif-1a* mice)

ii) The use of the iron chelator DFO and DFS and determine the effects of stabilising HIF-1α protein in β-*Hif-1a* mice on a high fat diet

iii) Knocking down *Hif-1a* in the MIN6 cell culture model

iv) Increasing HIF-1α protein in MIN6 cells by knocking down *Vhl*, or by transfecting with a mutant form of HIF-1α

These models will allow us to determine the role of HIF-1α in the β-cell and if the hypothesis proves correct, determine if increasing HIF-1α can be a viable option in the treatment of T2D.

4.3 Results

4.3.1 β-*Hif-1α* mice

4.3.1.1 Immunostaining in β-*Hif-1α* mice

Immunostaining was performed in WT (Figure 4.1) and β-*Hif-1α* mice (Figure 4.2) to confirm loss of HIF-1α protein in the β-cells. Positive HIF-1α is represented in red in the top left panel with the horizontal white arrows identifying HIF-1α and the vertical green arrows identifying HIF-1α in the blood vessels. Insulin is represented in green in the top right panel, nuclear staining (DAPI) represented in blue in the bottom left panel, and the merge of the three pictures in the bottom right panel. Figure 4.2 shows a lack of co-localisation of HIF-1α and insulin and thus no HIF-1α protein in the β-cells.

4.3.1.2 Glucose tolerance in β-*Hif-1α* mice

GTTs were performed in WT and β-*Hif-1a* mice to determine if there were any differences in glucose control. Glucose tolerance in WT mice were normal and importantly, in our colony, RIP-Cre mice also had normal glucose tolerance [260]. β-*Hif-1a* mice were fertile and did not differ in size or weight (data not shown). Both male and female β-*Hif-1a* mice had higher fasting glucose and male β-*Hif-1a* mice had significantly greater glucose excursions at 30 and 60 minutes post glucose loading compared to floxed control mice ($p < 0.05$, Figure 4.3 top panel). Female β-*Hif-1a* mice had significantly worse glucose tolerance at 60 minutes ($p < 0.05$, Figure 4.3 lower panel) and a trend to increased glucose at 60 minutes.

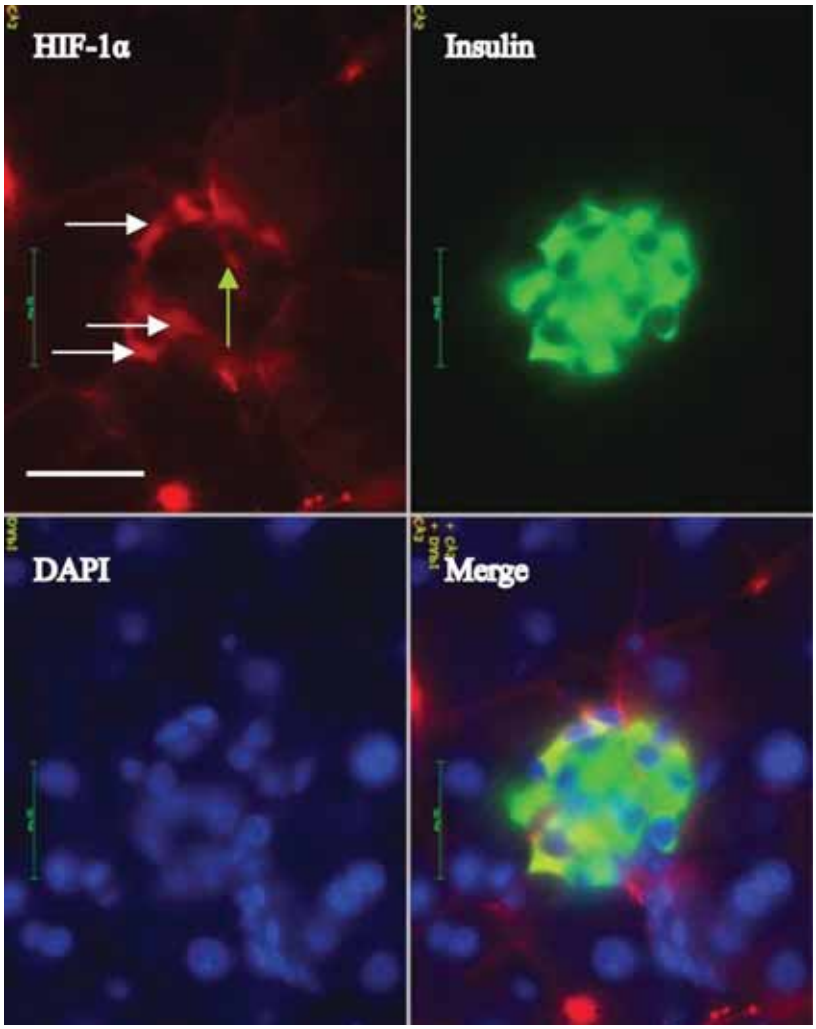

Figure 4.1 Fluorescent imaging of pancreatic sections from WT mice. HIF-1α is represented in the top left panel in red with the horizontal while arrows indicating positive HIF-1α staining while the vertical green arrow indicates positive HIF-1α in the blood vessel. Insulin is represented in the top right panel in green, nuclear staining in the bottom left panel shown in blue, and a merge of the three images in the bottom right panel. White scale bar: 20 μm.

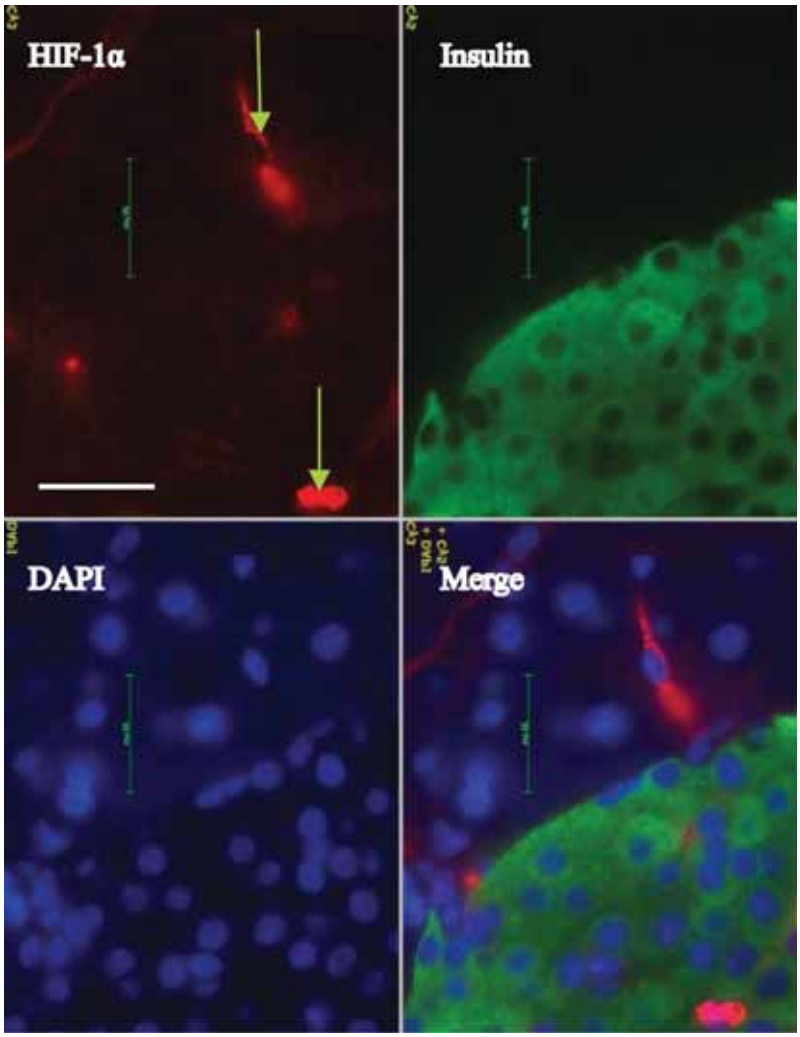

Figure 4.2 Fluorescent imaging of pancreatic sections from β-*Hif-1a* mice. HIF-1α is represented in the top left panel in red with the vertical green arrows indicating positive HIF-1α in the blood vessel. Insulin is represented in the top right panel in green, nuclear staining in the bottom left panel shown in blue, and a merge of the three images in the bottom right panel. White scale bar: 20 μm.

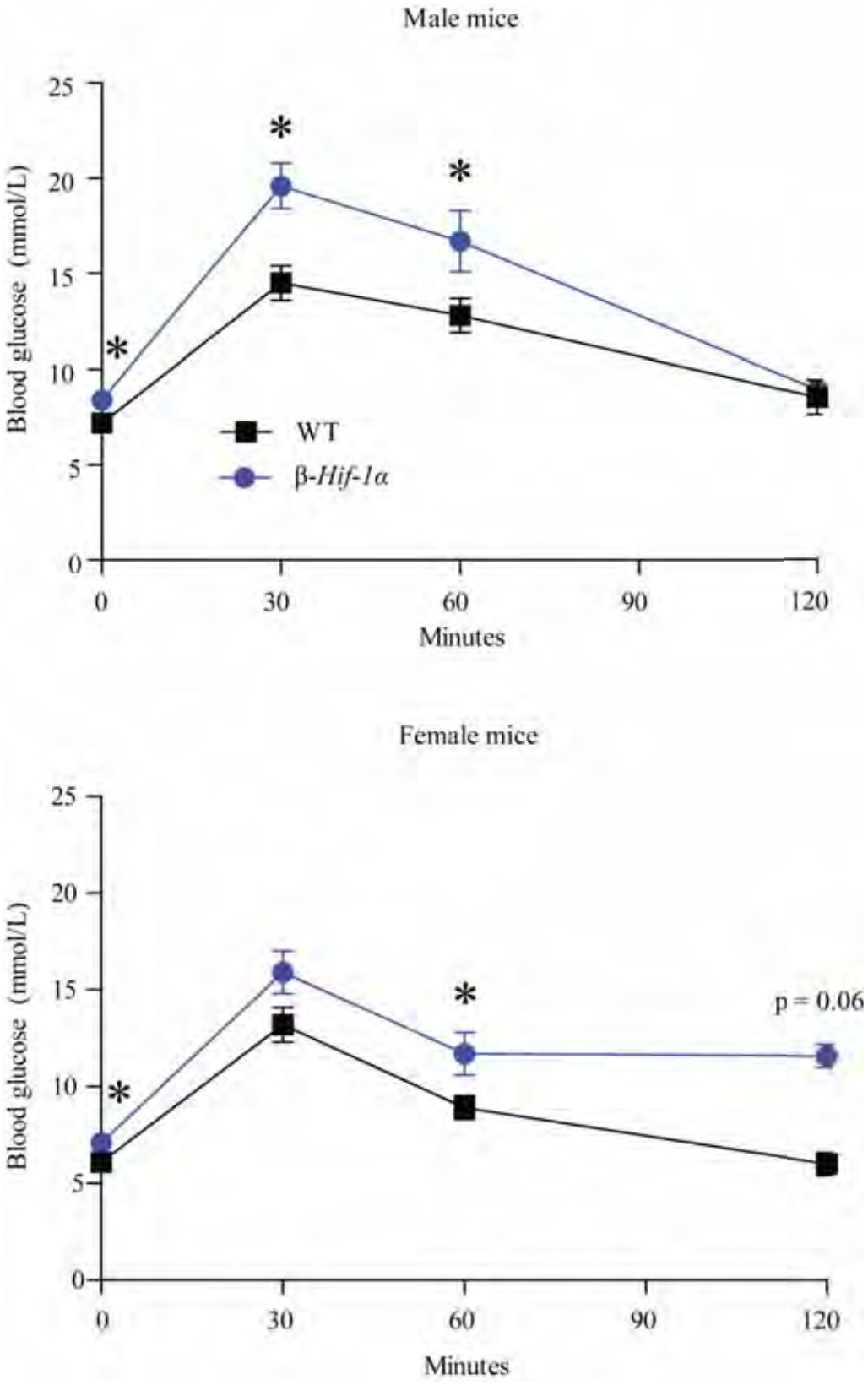

Figure 4.3 GTT in β-*Hif-1α* and WT mice. β-*Hif-1α* and WT mice were fasted for 16 hours and GTT performed after a glucose bolus in the form of an intraperitoneal injection of 20 % dextrose at a dose of 2 g/kg. n = 17 male WT, 25 male β-*Hif-1α*, 22 female WT, 22 female β-*Hif-1α*. * $p < 0.05$.

4.3.1.3 GSIS in β-*Hif-1α* mice

GSIS was performed in WT and β-*Hif-1a* mice to determine any changes in first phase insulin secretion. There were no significant differences in fasting insulin between both male and female WT and β-*Hif-1a* mice (Figure 4.4). However, as shown in the top panel of Figure 4.4, male β-*Hif-1a* mice had significantly reduced insulin at 2 and 5 minutes indicating impaired first phase GSIS ($p < 0.05$, Figure 4.4 top panel) while there was a trend to impairment in female β-*Hif-1a* mice.

4.3.1.4 GSIS in islets of β-*Hif-1α* mice

Islets were isolated from WT and β-*Hif-1a* mice and as expected, islets from WT mice had a dose dependent increase in GSIS with increasing amounts of glucose stimulation (Figure 4.5). However, consistent with the *in vivo* effects, islets isolated from β-*Hif-1a* mice had impaired GSIS (> 80 % reduction at 3.3 and 11 mM glucose; Figure 4.5). The difference was not statistically significant at 22 mM glucose.

4.3.1.5 Total insulin content and β-cell mass in β-*Hif-1α* mice

To determine whether differences observed in glucose tolerance and GSIS were due to differences in the β-cell, we examined total insulin content and β-cell mass in both WT and β-*Hif-1a* mice. There were no significant differences in β-cell mass between islets in WT mice compared to β-*Hif-1α* mice (Figure 4.6) nor were there any significant differences in total insulin content [260].

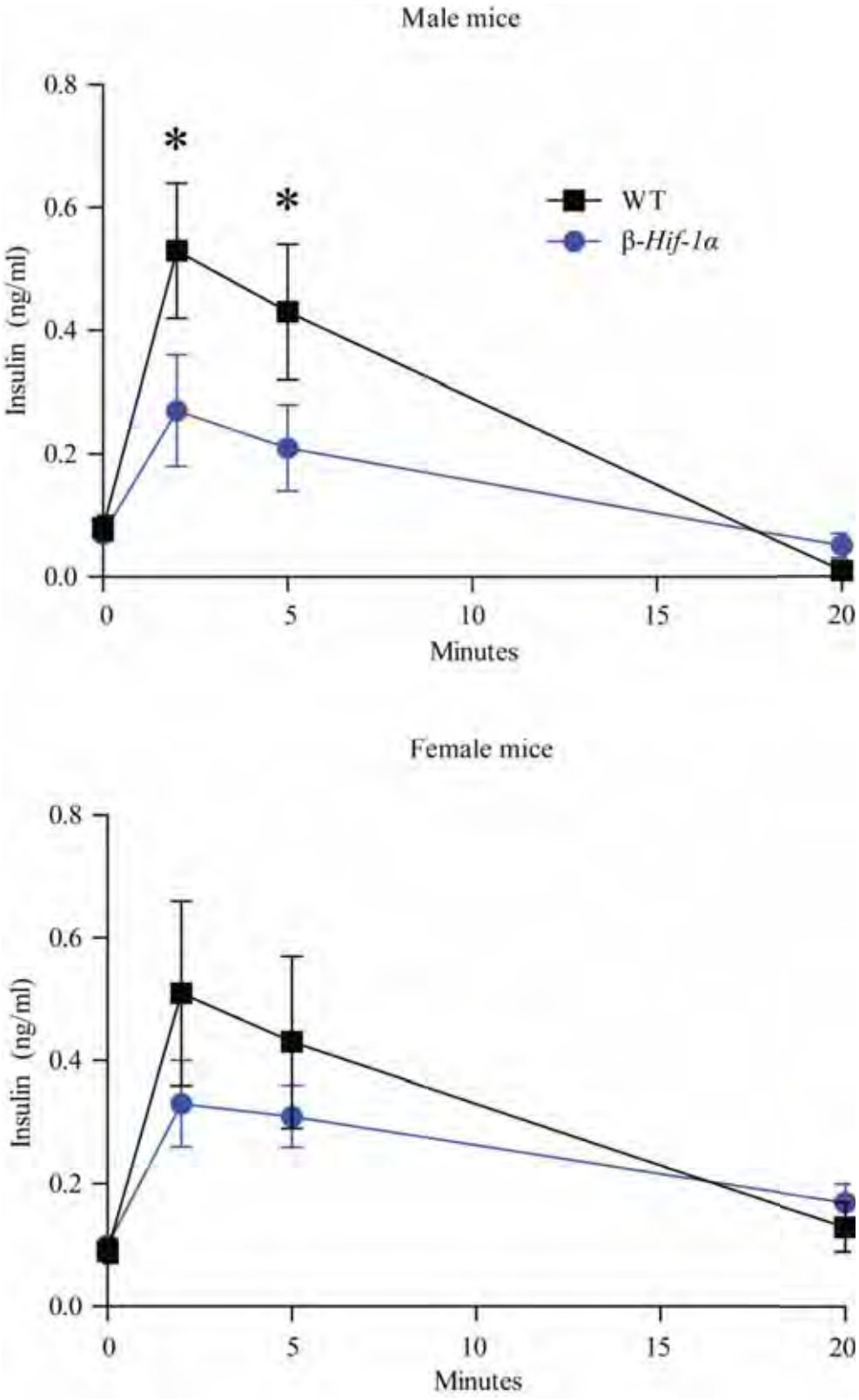

Figure 4.4 GSIS in β-*Hif-1α* and WT mice. β-*Hif-1α* and WT mice were fasted for 16 hours and GSIS performed after a glucose bolus in the form of an intraperitoneal injection of 20 % dextrose at a dose of 2 g/kg. * $p < 0.05$.

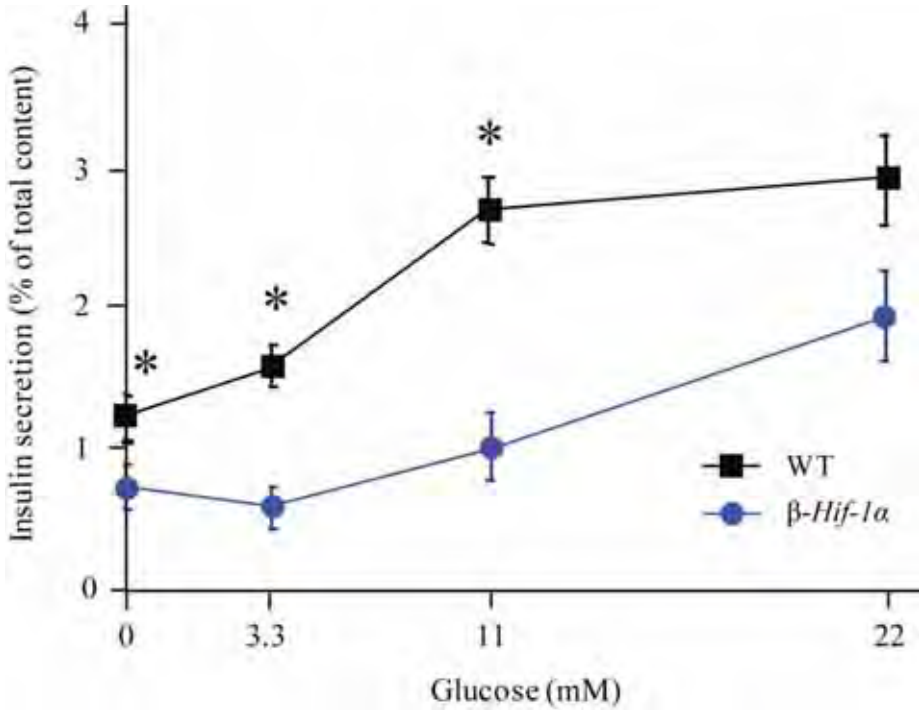

Figure 4.5 GSIS in islets isolated from β-*Hif-1α* and WT mice. Islets were isolated from β-*Hif-1α* and WT mice and GSIS was measured. * $p < 0.05$.

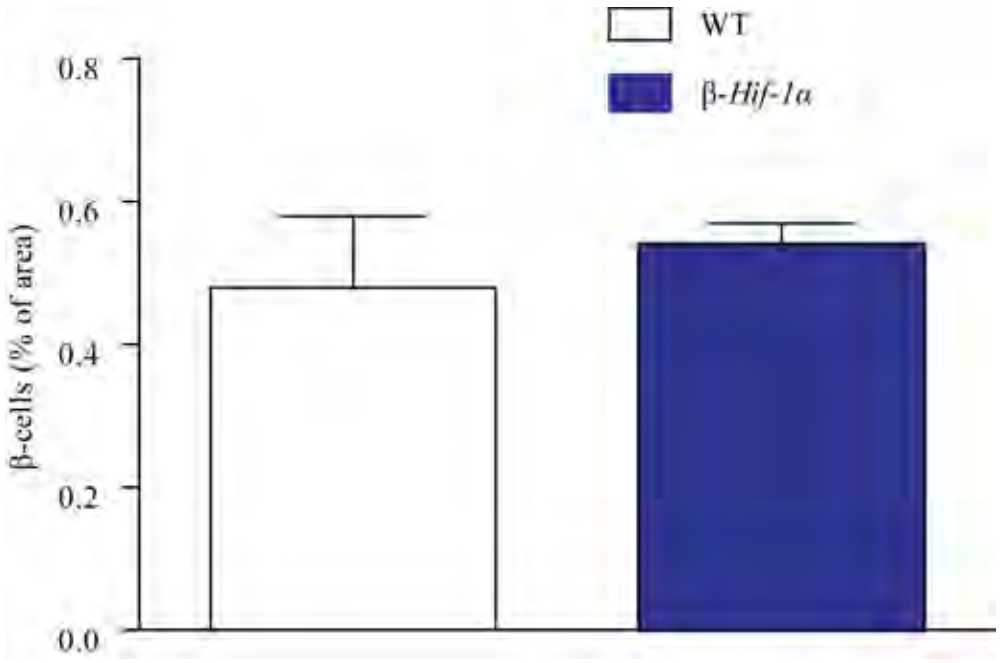

Figure 4.6 β-cell mass in WT and β-*Hif-1α* mice. Pancreas sections were cut and stained with insulin. β-cell mass was determined as the percentage of positive insulin staining as a proportion of the total pancreatic area.

4.3.1.6 Intracellular ATP content in islets of β-*Hif-1α* mice

As expected, islets from WT mice had a 60 % increase in intracellular ATP content after exposure to 25 mM glucose ($p < 0.05$, Figure 4.7). Islets from β-*Hif-1a* mice had significantly decreased intracellular ATP content at 1 mM glucose (60 % decrease, $p < 0.05$) and the increase in intracellular ATP content with 25 mM glucose was severely blunted.

4.3.1.7 Gene expression changes in islets of β-*Hif-1α* mice

β-*Hif-1a* islets had a greater than 40 % decrease in the glucose transporter *Glut2*, key glycolytic genes *Gck*, *G6pi*, *PFK*, *phosphoglycerate mutase 2* (*Pgam2*), and the MODY transcription factor *Hnf4a*. There was also a significant reduction in the insulin signalling gene *RAC-β serine/threonine protein kinase* (*Akt2*, $p < 0.05$, Figure 4.8).

4.3.2 DFS and β-*Hif-1α* mice

4.3.2.1 GTT in β-*Hif-1α* mice on a HFD

In a separate cohort of mice (Figure 4.9), we performed GTT and replicated the finding of impaired glucose tolerance, with significantly higher glucose at 30 minutes ($p < 0.05$). For β-*Hif-1a* mice, weight was 24.7 ± 0.6 g, and for controls, weight was 25.2 ± 0.6 g (data not shown, $p > 0.05$). To examine β-cell compensation in the setting of insulin resistance, we placed mice on HFD (45 % of calories from fat) for three weeks. Weight increased to 29.4 ± 0.9 g for β-*Hif-1a* mice and to 29.0 ± 0.8 g for controls (data not shown, $p > 0.05$). Glucose tolerance deteriorated in WT mice (Figure 4.10, black line) but more severely in β-*Hif-1a* mice (Figure 4.11, black line) as shown by the area under the curve (AUC, $p = 0.047$ for WT HFD fed versus β-*Hif-1a* HFD fed mice, Figure 4.12).

4.3.2.2 GTT in β-*Hif-1α* mice on a HFD + DFS

DFS is the oral analogue of DFO and is an iron chelator used in our laboratory to prevent proteasomal degradation of HIF-1α under normoxic conditions. Following three weeks of HFD, all mice were changed to HFD mixed with DFS (HFD + DFS) to increase HIF-1α protein. After three weeks, weight was 29.0 ± 0.8 g in β-*Hif-1a* mice versus 29.0 ± 0.8 g in controls (data not shown, $p > 0.05$). As shown by the dotted line in Figure 4.10, despite continuing HFD, WT mice had highly significantly improved glucose tolerance ($p < 0.05$). In contrast, there was no improvement in β-*Hif-1a* mice ($p > 0.05$, Figure 4.11), demonstrating that β-cell HIF-1α was required for DFS to improve glucose tolerance. AUCs are shown in Figure 4.12.

4.3.2.3 β-cell mass in control and β-*Hif-1α* mice after HFD + DFS

To determine whether there were any gross changes in the β-cells, β-cell mass was measured. β-*Hif-1a* mice had 69 % greater β-cell mass than controls at the end of the study ($p < 0.05$, Figure 4.13), despite worse glucose tolerance, suggesting attempted and unsuccessful β-cell compensation.

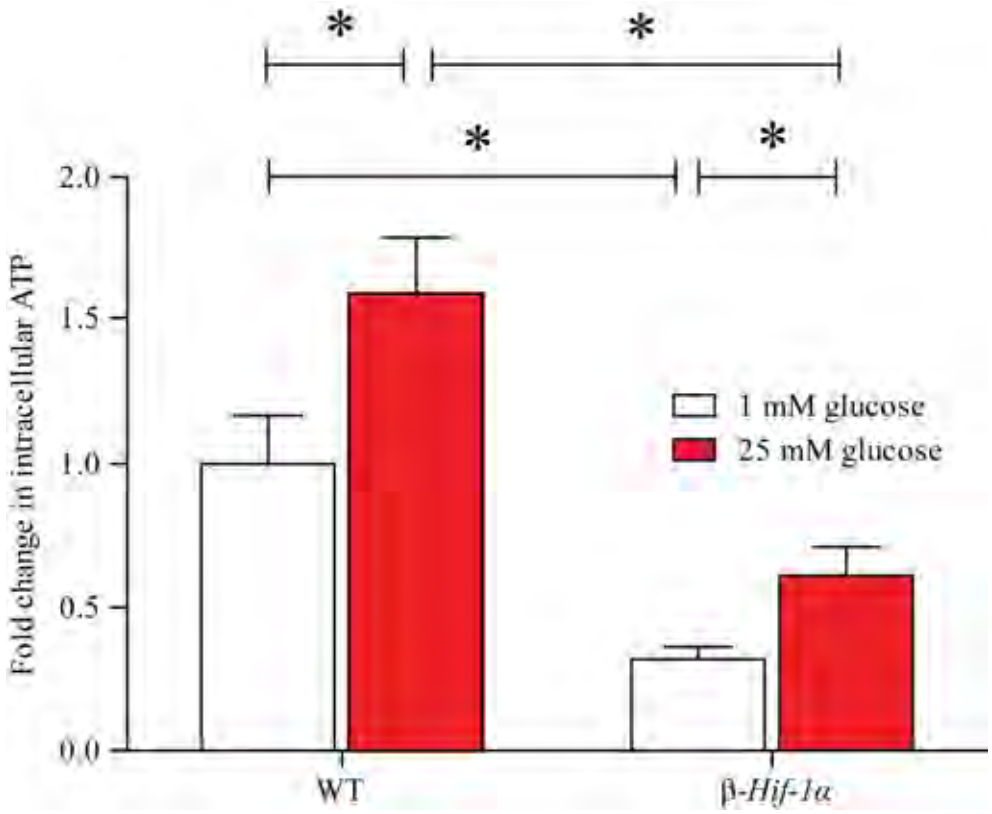

Figure 4.7 Intracellular ATP content in WT and β-*Hif-1α* islets. Islets were isolated from WT and β-*Hif-1α* mice and intracellular ATP content measured. * $p < 0.05$.

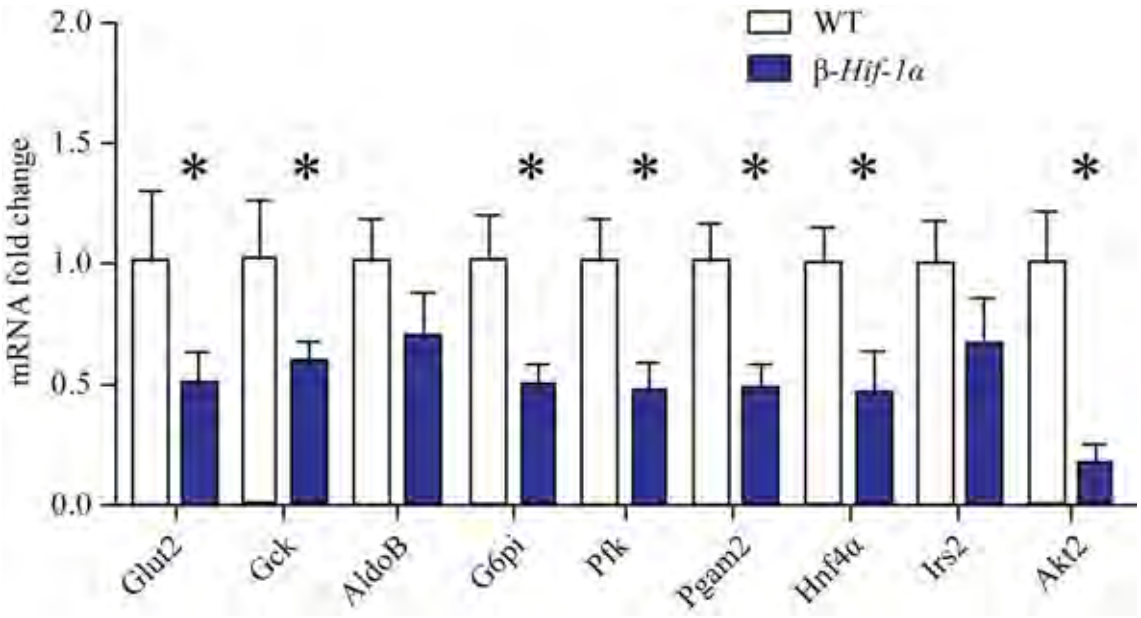

Figure 4.8 RT-PCR in islets isolated from WT and β-*Hif-1α* mice. Islets were isolated from WT and β-*Hif-1α* mice and gene expression analysed by RT-PCR. * $p < 0.05$ compared to control.

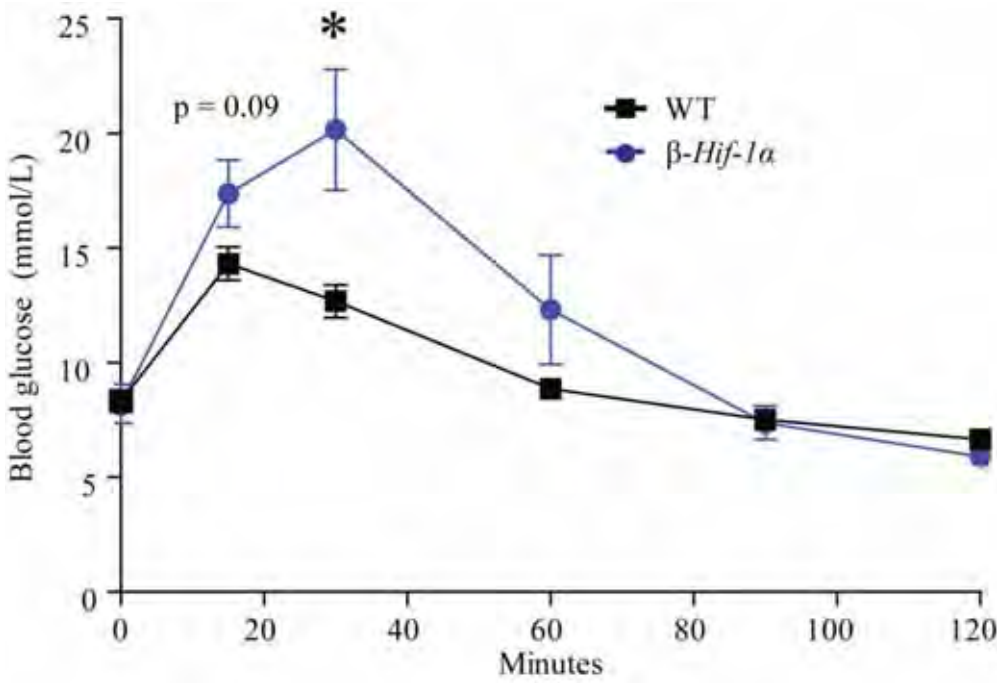

Figure 4.9 GTT in WT and β-*Hif-1α* mice. WT and β-*Hif-1α* mice were fasted for 16 hours and GTT performed after a glucose bolus in the form of an intraperitoneal injection of 20 % dextrose at a dose of 2 g/kg. * $p < 0.05$.

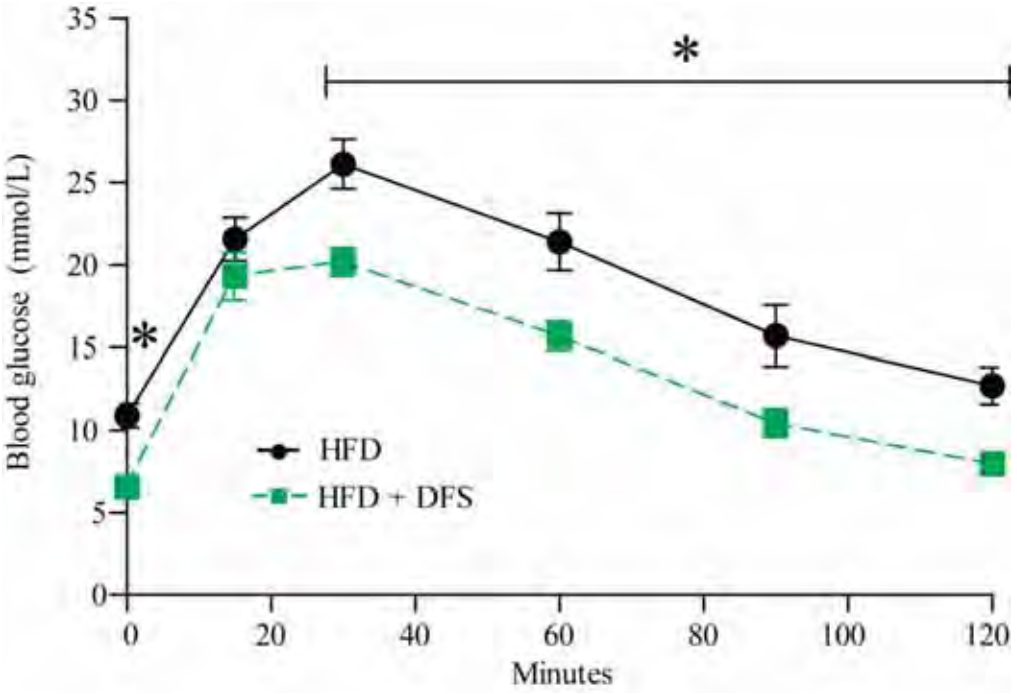

Figure 4.10 GTT in WT mice on a HFD with and without DFS. WT were fasted for 16 hours and GTT performed after a glucose bolus in the form of an intraperitoneal injection of 20 % dextrose at a dose of 2 g/kg. DFS was mixed into the HFD at a dose of 30 mg/kg/day. * $p < 0.05$.

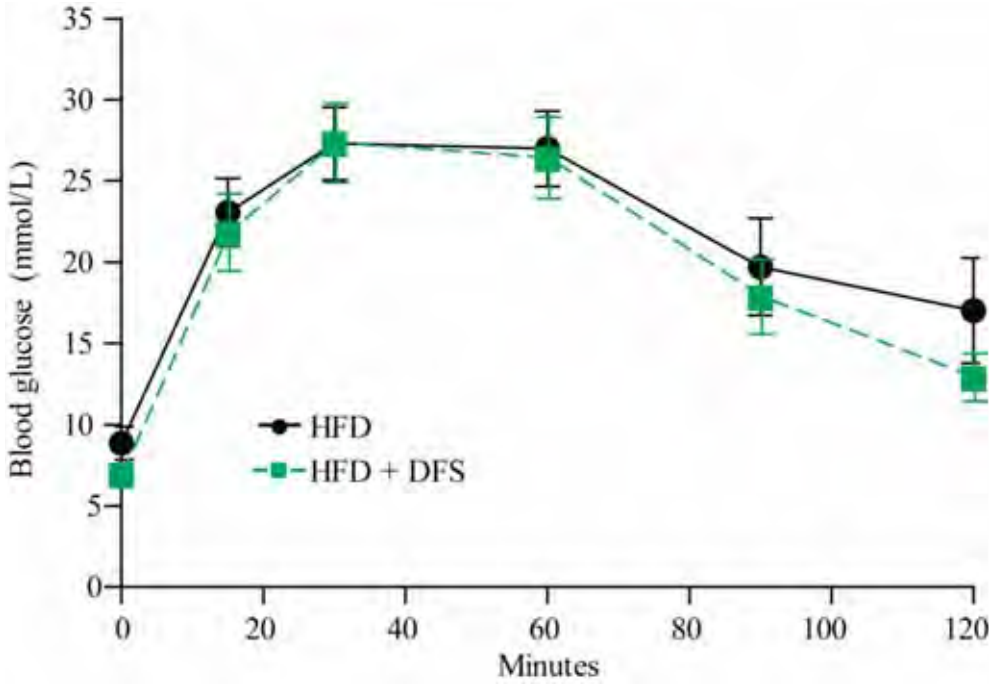

Figure 4.11 GTT in β-*Hif-1α* mice on a HFD with and without DFS. β-*Hif-1α* mice were fasted for 16 hours and GTT performed after a glucose bolus in the form of a intraperitoneal injection of 20 % dextrose at a dose of 2 g/kg. DFS was mixed into the high fat diet at a dose of 30 mg/kg/day.

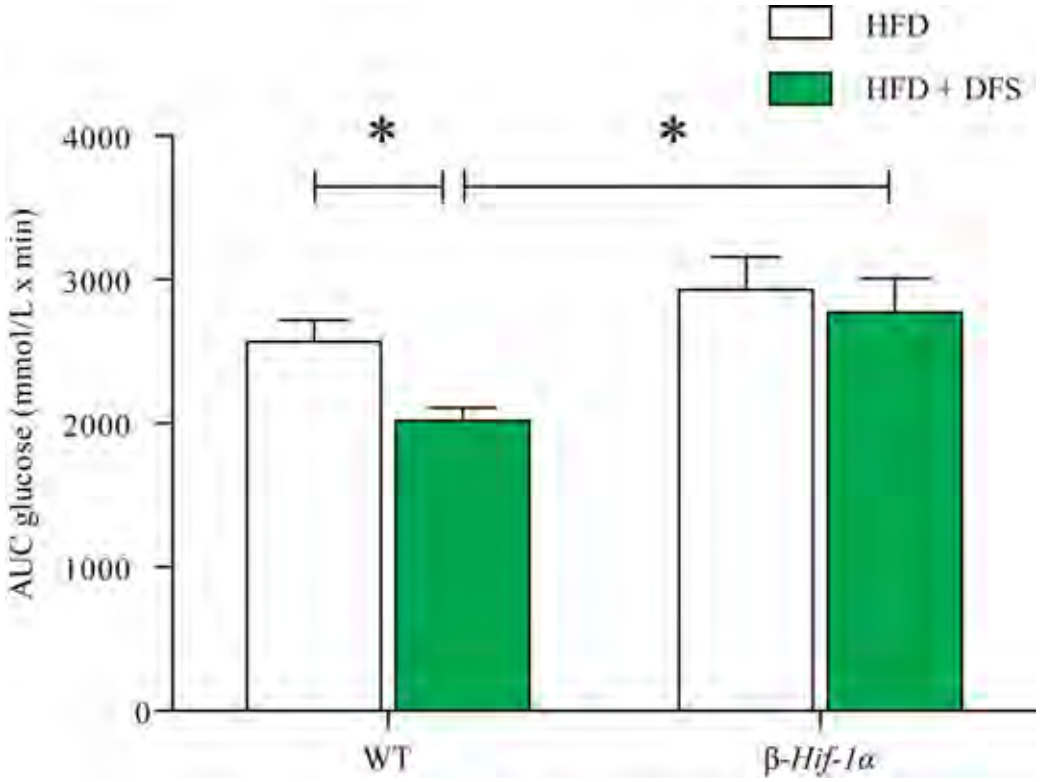

Figure 4.12 AUC for WT and β-*Hif-1α* GTT on a HFD and HFD + DFS. AUC was calculated using the values from Figures 4.10 and 4.11. * $p < 0.05$.

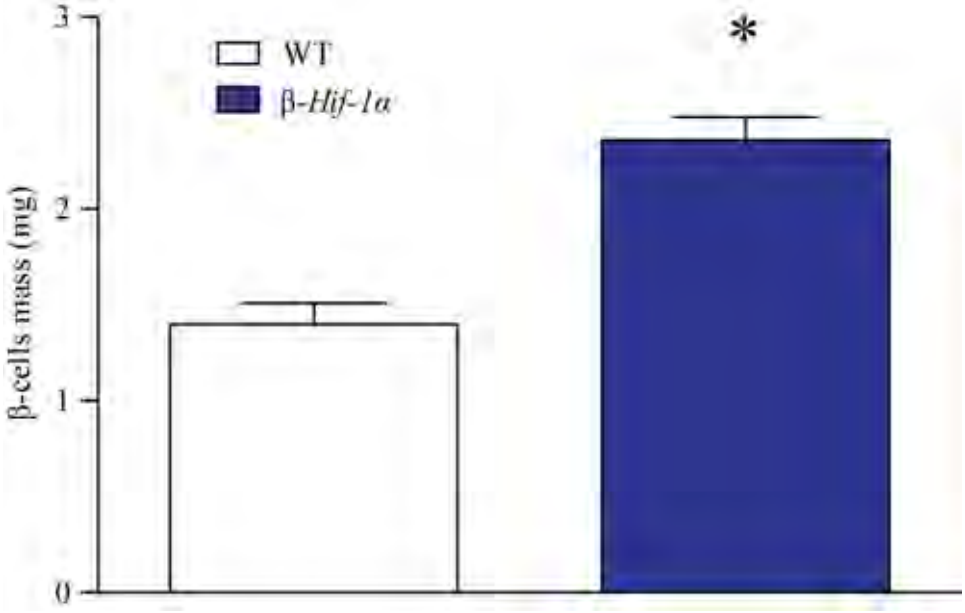

Figure 4.13 β-cell mass in WT and β-*Hif-1α* mice. Pancreas sections were cut and stained with insulin. β-cell mass was determined as the percentage of positive insulin staining as a proportion of the total pancreatic weight. * $p < 0.05$.

4.3.3 MIN6 cells and HIF-1α

4.3.3.1 Gene expression in MIN6 cells with knockdown of *Hif-1α*

MIN6 cells transfection with siRNA achieved approximately 70 % knockdown of *Hif-1a* mRNA (Figure 4.14). This led a decreased expression of glucose transporter and glycolytic gene expression, including *Gck*, *Glut2*, *G6pi*, *AldoB*, and *Pfk* (40 % – 60 %; Figure 4.15). These changes were similar to those for β-*Hif-1a* islets (Figure 4.8).

4.3.3.2 Insulin secretion in MIN6 cells with knockdown of *Hif-1α*

MIN6 cells transfected with control siRNA had a greater than 2-fold increase in GSIS at 11 mM and 22 mM glucose (Figure 4.16). However, MIN6 cells that had a knockdown of *Hif-1α* had markedly impaired GSIS at both 11 mM and 25 mM glucose ($p < 0.05$) and no significant increases from 1 mM glucose. Insulin secretion using the secretagogue KCl was also impaired in MIN6 cells with a knockdown of *Hif-1α*, with an approximate 25 % decrease from control cells ($p < 0.05$, Figure 4.16)

4.3.3.3 Intracellular ATP content in MIN6 with knockdown of *Hif-1α*

MIN6 cells transfected with control siRNA had a significant increase in intracellular ATP content when stimulated from 1 to 25 mM glucose ($p < 0.05$, Figure 4.17). Knockdown of *Hif-1α* in MIN6 cells severely inhibited ATP generation, with intracellular ATP content significantly decreased compared to the respective control values ($p < 0.05$, Figure 4.17).

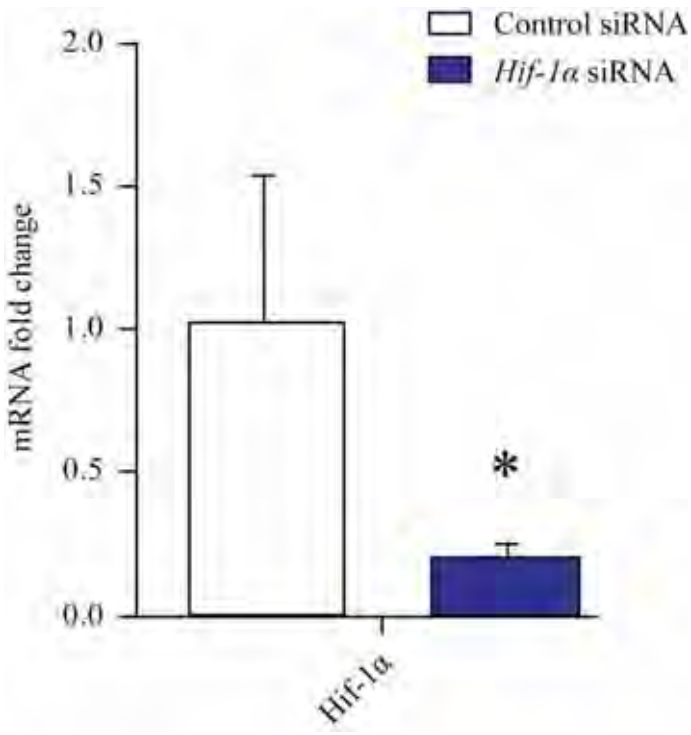

Figure 4.14 RT-PCR in MIN6 with knockdown of *Hif-1α*. MIN6 cells were transfected with control and *Hif-1α* siRNA and gene expression analysed by RT-PCR. * $p < 0.05$ compared to control.

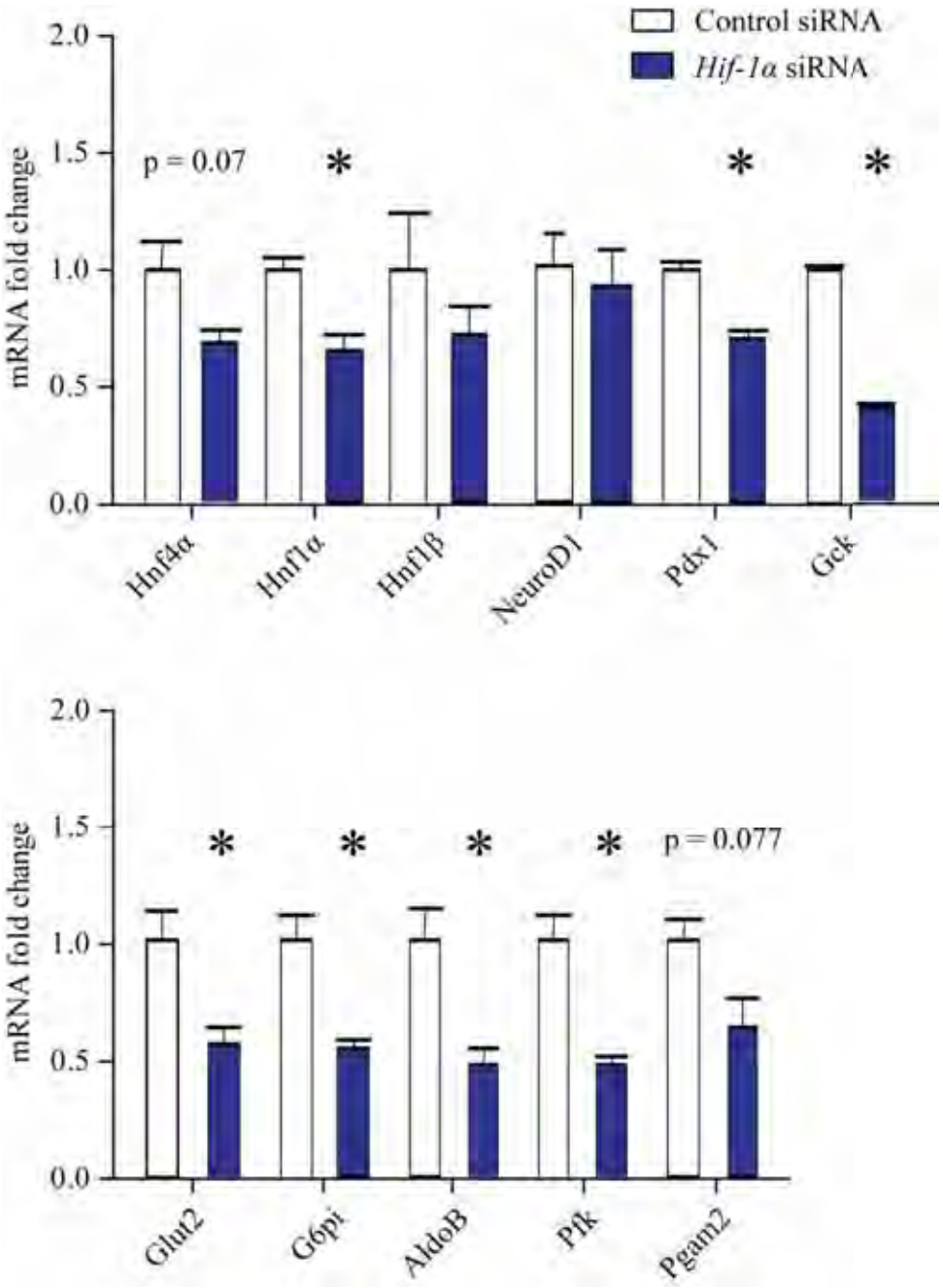

Figure 4.15 RT-PCR in MIN6 cells with knockdown of *Hif-1α*. MIN6 cells were transfected with control and *Hif-1α* siRNA and gene expression analysed by RT-PCR. * $p < 0.05$ compared to control.

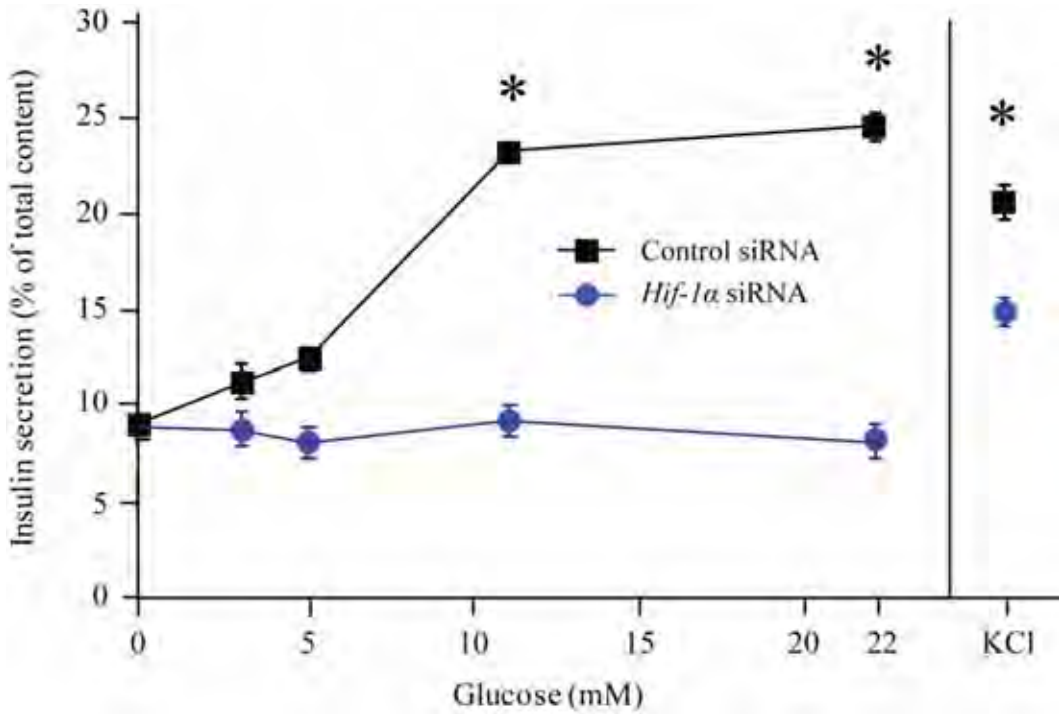

Figure 4.16 GSIS in MIN6 cells with knockdown of *Hif-1α*. MIN6 cells were transfected with control and *Hif-1α* siRNA and GSIS was measured. * $p < 0.05$.

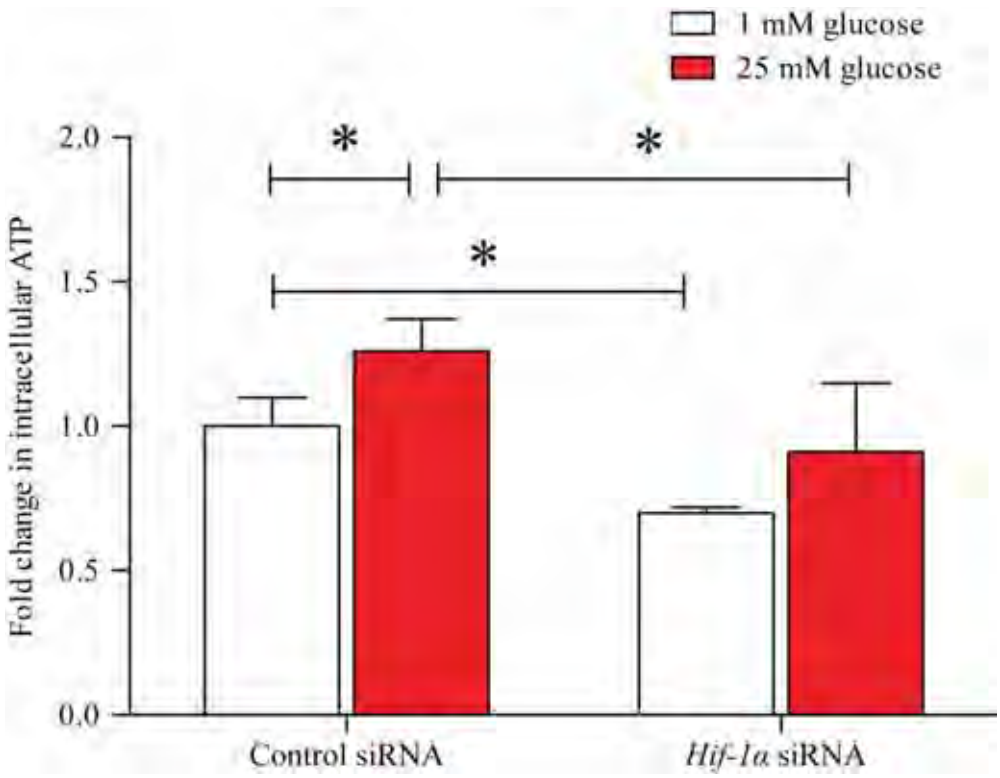

Figure 4.17 Intracellular ATP content in MIN6 cells with knockdown of *Hif-1α*. MIN6 cells were transfected with siRNA to knockdown *Hif-1α* and intracellular ATP measured and expressed as a fold change compared to control basal levels. * $p < 0.05$.

4.3.3.4 MIN6 cells with knockdown of *Vhl*

MIN6 cells were transfected with two doses of *Vhl* siRNA, low and high dose, to induce varying degrees of *Vhl* knockdown. Transfection with low dose *Vhl* siRNA led to a decrease in *Vhl* mRNA by 33 % ($p < 0.05$, Figure 4.18). This led to a significant increase in *Glut2,* decrease in *Akt2* ($p < 0.05$, Figure 4.18), and a non significant increase in GSIS (Figure 4.19). By doubling the siRNA concentration (high dose), approximately 55 % *Vhl* knockdown was achieved. This led to decreased *Akt2* expression and the increase in *Glut2* was lost ($p < 0.05$, Figure 4.20). This was accompanied by a non significant impairment in GSIS ($p = 0.17$, Figure 4.21).

4.3.3.5 MIN6 cells transfected with a mutant form of HIF-1α

Under normoxic conditions, HIF-1α only has a half life of several minutes due to proteasomal degradation. In order to successfully overexpress HIF-1α under normoxic conditions, a mutant form of HIF-1α was used. This mutant HIF-1α had proline to alanine mutations at positions 402 and 577, making it resistant to the prolyl hydroxylases. Increasing HIF-1α levels by transient transfections with this mutant form of HIF-1α caused significant impairment in GSIS ($p < 0.05$, Figure 4.22). This was associated with the expected increases in *Hif-1a* (> 35-fold) and *Glut1* (> 3-fold, Figure 4.23). However, there was also a significant decrease in expression of *Gck* ($p < 0.05$, Figure 4.23). Interestingly, there was significantly decreased total insulin content in the mutant HIF-1α transfected cells (approximately 50 % of vector transfected, Figure 4.24). The transfection efficiency of this mutant form of HIF-1α is unknown; however previous transfections with labelled siRNA have shown a transfection efficiency of approximately 70 % by FACS analysis (data not shown).

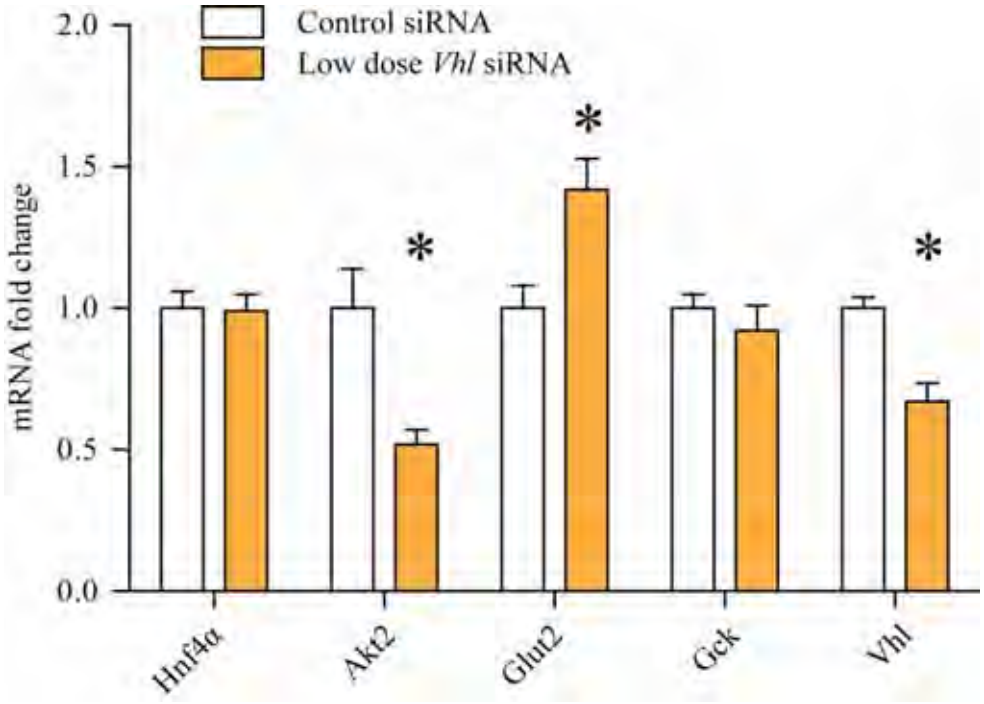

Figure 4.18 RT-PCR in MIN6 cells with low dose knockdown of *Vhl*. MIN6 cells were transfected with a low dose of *Vhl* siRNA and gene expression measured. * $p < 0.05$.

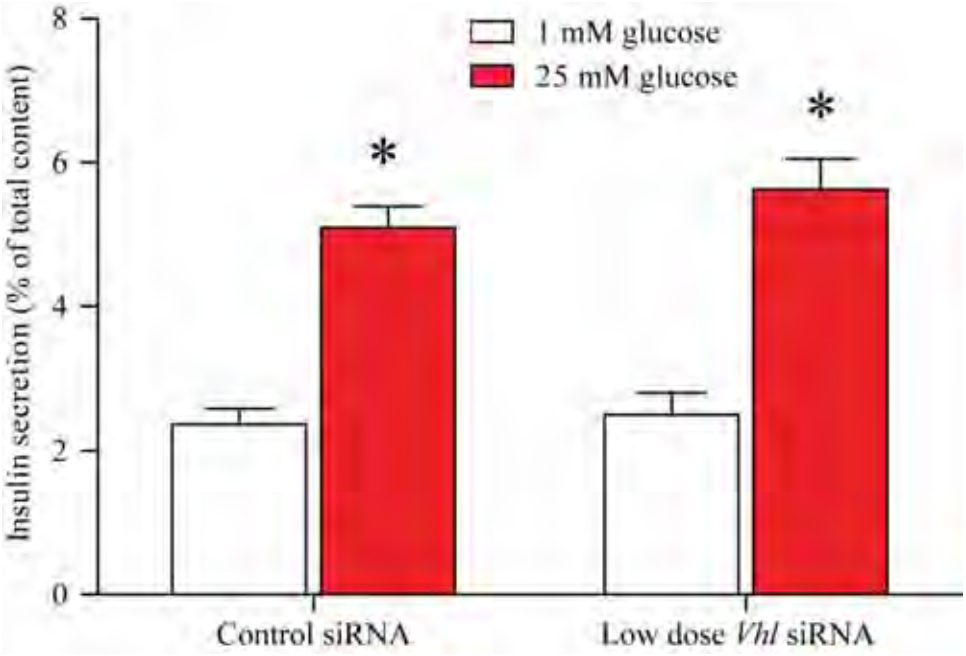

Figure 4.19 GSIS in MIN6 cells with low dose knockdown of *Vhl*. MIN6 cells were transfected with a low dose of *Vhl* siRNA and GSIS measured. * $p < 0.05$ compared to their relative basal levels.

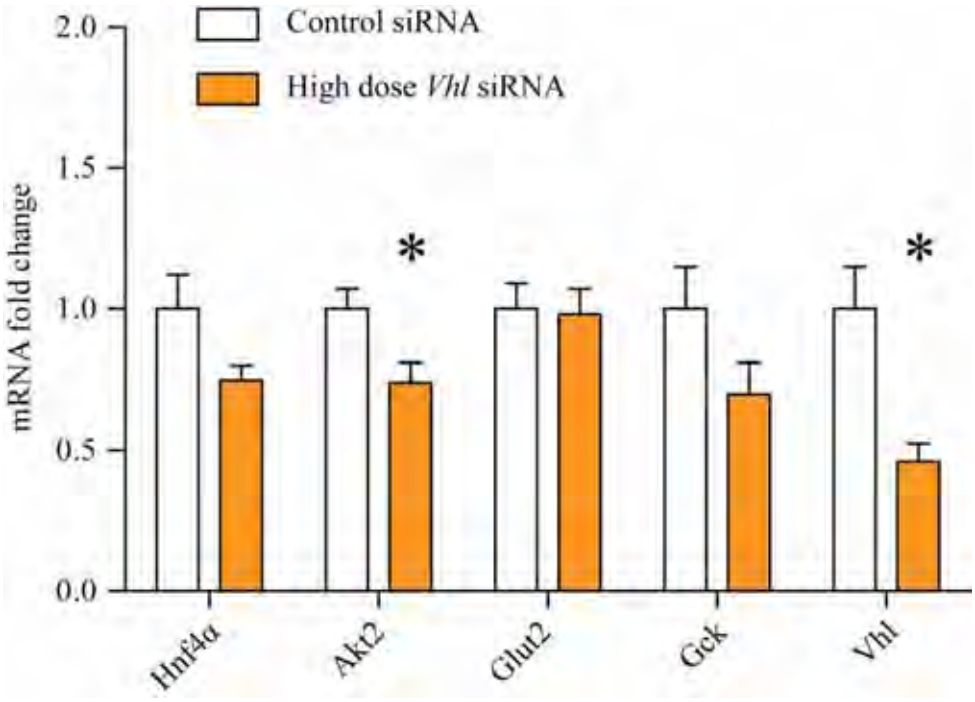

Figure 4.20 RT-PCR in MIN6 cells with high dose knockdown of *Vhl*. MIN6 cells were transfected with a high dose of *Vhl* siRNA and gene expression measured. * $p < 0.05$.

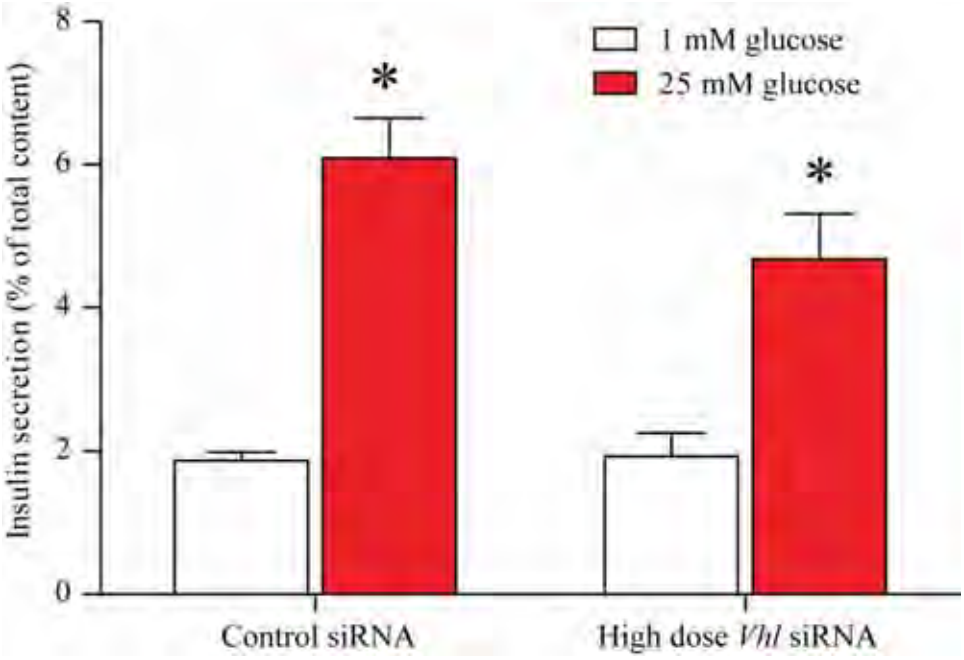

Figure 4.21 GSIS in MIN6 cells with high dose knockdown of *VHL*. MIN6 cells were transfected with a high dose of *VHL* siRNA and GSIS measured. * $p < 0.05$ compared to their relative basal levels.

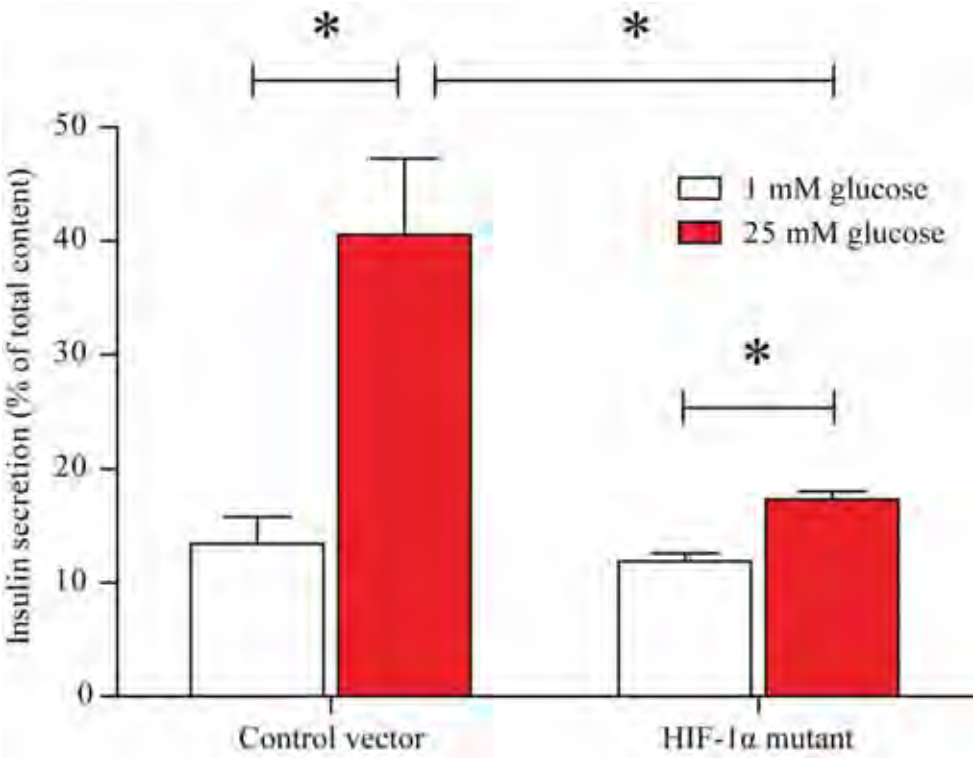

Figure 4.22 GSIS in MIN6 cells transfected with a HIF-1α mutant. MIN6 cells were transfected with a dual P402A and P577A HIF-1α mutant and GSIS measured. * $p < 0.05$.

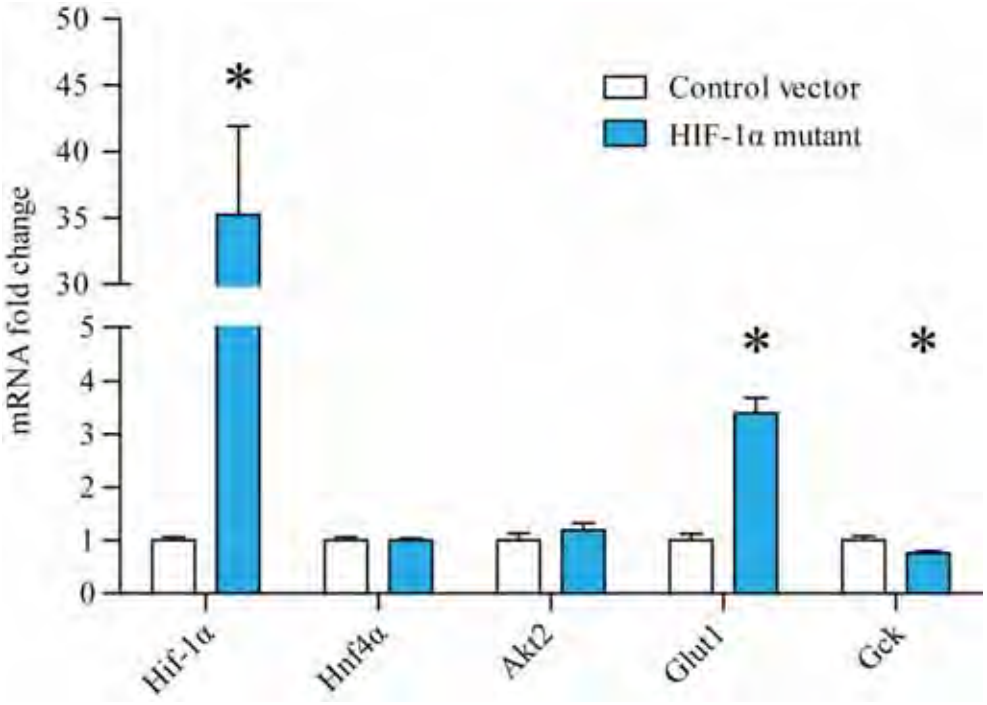

Figure 4.23 RT-PCR in MIN6 cells transfected with the HIF-1α mutant. MIN6 cells were transfected with a dual P402A and P577A HIF-1α mutant and gene expression analysed by RT-PCR. * $p < 0.05$.

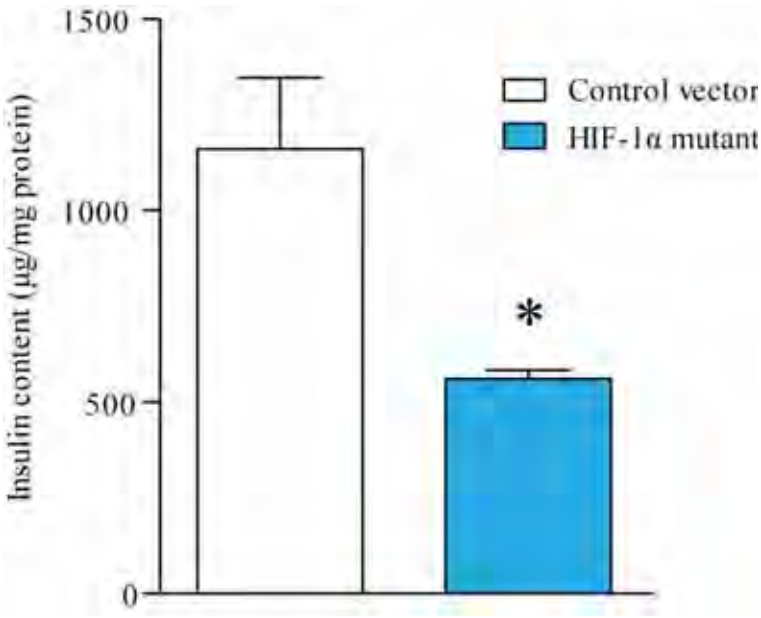

Figure 4.24 Total insulin content in MIN6 cells transfected with the HIF-1α mutant. MIN6 cells were transfected with a dual P402A and P577A HIF-1α mutant and total insulin content measured. * $p < 0.05$ compared to control.

4.3.3.6 MIN6 cells treated with ferric citrate

As we had studied the effects of removing intracellular iron by using the iron chelator DFS, we studied the effects of introducing exogenous iron by using ferric citrate. At high doses (0.1 mg/ml), there was obvious cell death. At lower doses (0.03 mg/ml), there were significant decreases in *Hif-1a*, *Hnf4a*, *Glut1*, and *Glut2* expression ($p < 0.05$, Figure 4.25). This identifies iron as a potential regulator of *Hif-1a* in β-cells. There was also significantly impaired GSIS, with only a non significant 13 % increase, following high glucose in iron-treated cells ($p = 0.17$ versus control high glucose, data not shown).

4.3.3.7 Cell cycle progression with DFO

To determine whether DFO had any effect on cell cycle progression, flow cytometry analysis of BrdU incorporation and 7AAD staining in was performed. There were no significant changes in any of the cell cycle phases, with only a small but non significant increase in the G2/M phase with DFO treatment (19.6 % vs. 18.1 %, $p = 0.08$, Figure 4.26).

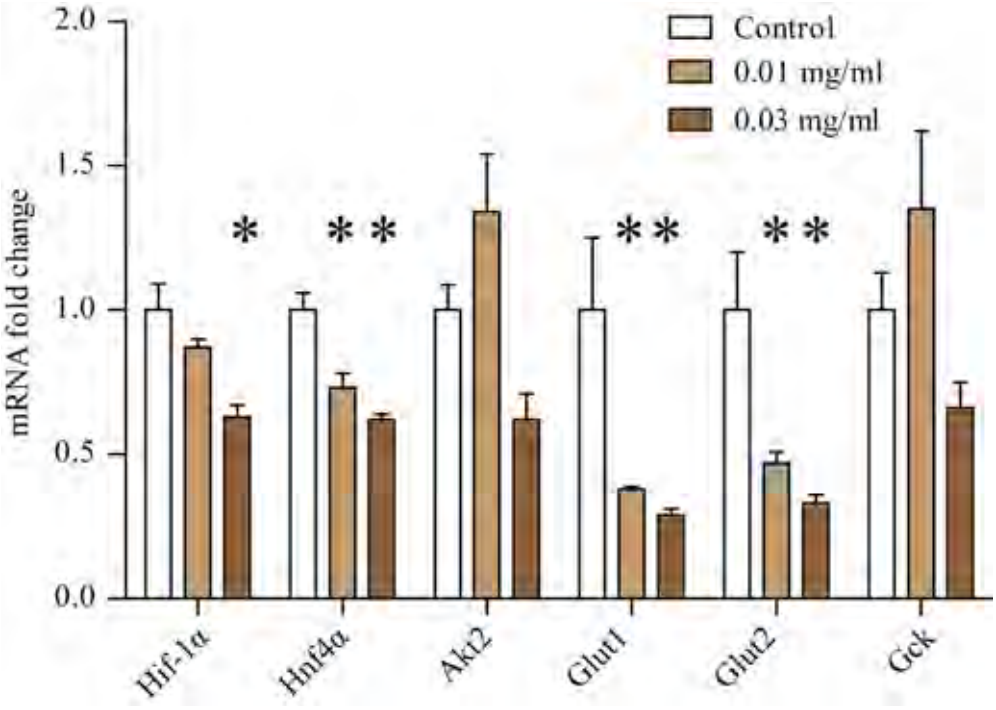

Figure 4.25 RT-PCR in MIN6 cells treated with ferric citrate. MIN6 cells were treated with different concentrations of ferric citrate and gene expression analysed by RT-PCR. * $p < 0.05$ compared to control.

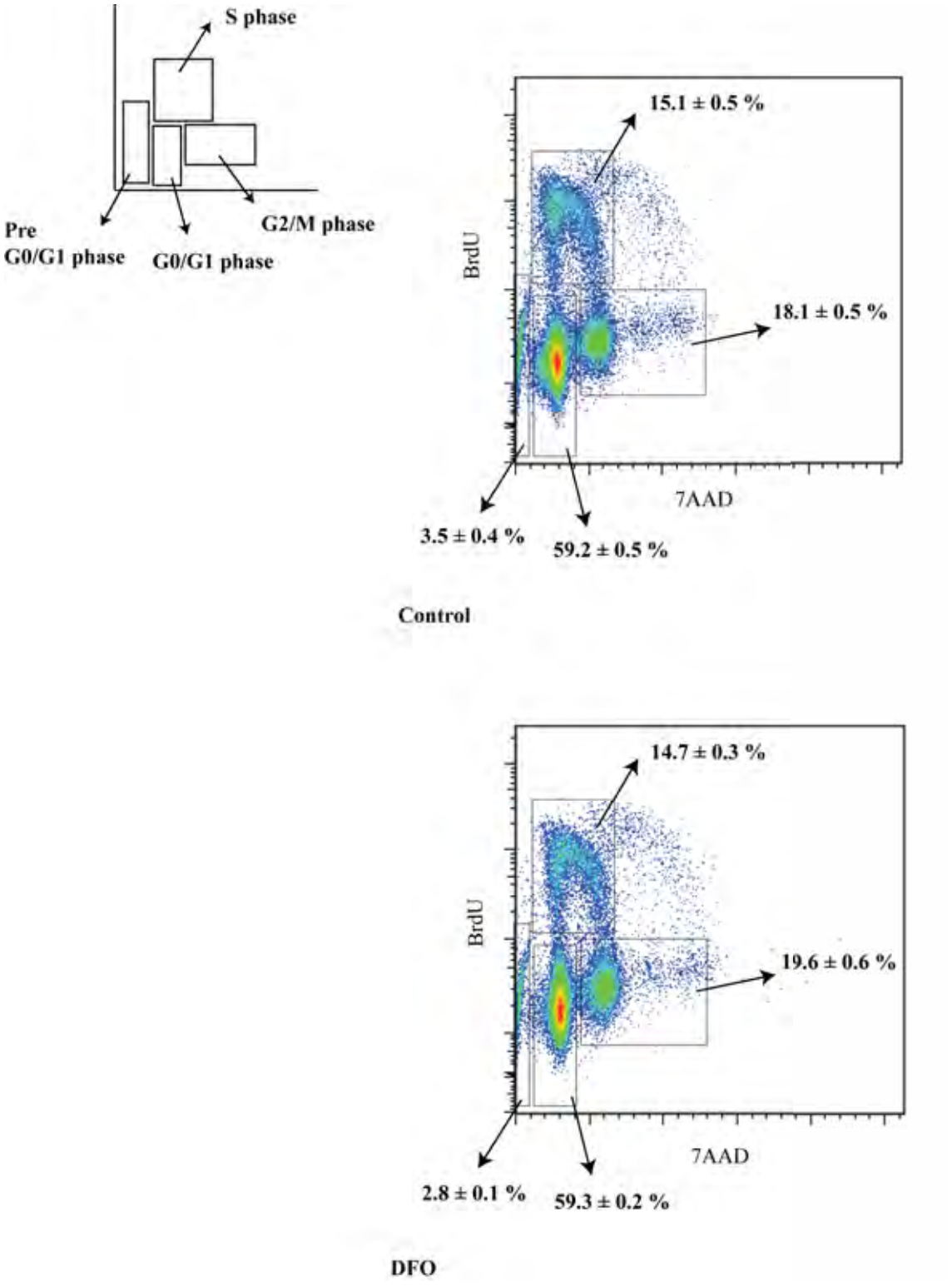

Figure 4.26 Analysis of cell cycling in MIN6 cells with and without DFO treatment. MIN6 cells were treated with 125 μM DFO cells and were stained with BrdU and 7AAD. Cells were sorted by fluorescent automated cell sorting (FACS) and analysed using FlowJo (Tree Star).

4.4 Discussion

People with T2D characteristically have pronounced impairment of first-phase insulin secretion [272-274]. This defect is intrinsic, as it persists in isolated islets [74, 327] and is relatively glucose specific [74, 328] in the earlier stages of the disease. In previous studies, Gunton et al. demonstrated that T2D islets had decreased *ARNT* expression and, using gene inactivation approaches, showed that this could contribute to altered β-cell function. In the present study, we show that T2D islets had decreased HIF-1α [260], which we show is also important for islet function. Deletion of HIF-1α in C57BL/6 mice resulted in impaired ATP generation and impaired glucose tolerance, accompanied by altered gene expression. Similar results were found in MIN6 cells using siRNA. The β-cell defect was relatively glucose specific, with only approximately 25 % impairment in KCl-stimulated insulin release.

Although HIF-1α protein is tightly regulated, several methods of increasing it exist. These include hypoxia, decreasing VHL protein, mutation or decreased expression of the prolyl hydroxylases, treatment with heavy metals such as cobalt chloride, and iron chelation. Severe hypoxia and cobalt chloride are toxic. Genetic modification is not usually a therapeutic option for humans, although the future possibility exists with antisense RNA strategies. Thus, we studied the effects of iron chelation with DFS or DFO.

Treating mice made diabetic by high-fat feeding with DFS improved glucose tolerance. DFS was also effective in C57BL/6 and Balb/c wild-type mice [260] but was completely ineffective in mice lacking β-cell HIF-1α, demonstrating that HIF-1α is required for the benefit. Our HFD had 45 % of calories from fat, compared with 12 % in normal chow. While this is high, the average fat intake in the American diet is more than 30 %. The top 20 % of the population consume 46 % of calories from fat [22, 23].

DFS was effective despite continuing the HFD, suggesting that it may be effective in people with T2D, in whom high fat-intake is common.

Surprisingly, DFO treatment normalised *ARNT* and other genes in T2D islets [260]. HIF-1α is predominantly regulated at the protein level, and DFO treatment was apparently sufficient to normalise HIF-1α function, as assessed by expression of downstream genes. The magnitude of effect on *ARNT*, *HNF4A*, and *G6PI* was large in T2D islets (> 10-fold), in which basal HIF-1α was low [260]. This is the first time that a strong regulator of *ARNT* expression has been identified. In contrast, the change in *ARNT* expression in normal islets, in which HIF-1α was not low at baseline was modest. *Hif-1a* itself was decreased by treatment with iron.

In other cell types, HIF-1α regulates *PDK1* [122, 241], *COX4.1*, *COX4.2*, and LON protease changes associated with increased ATP [131]. Consistent with these studies, we observed that decreasing HIF-1α decreased ATP. ATP generation is required in β-cells for sensing of glucose, which in turn triggers insulin release. Furthermore, impaired ATP generation in our models was associated with impaired insulin release, consistent with HIF-1α being a regulator of β-cell energy homeostasis and insulin release. Thus, decreased HIF-1α impaired glucose stimulated ATP generation, providing the mechanism by which decreased availability of a transcription factor can cause β-cell dysfunction.

Recently, four groups reported clear, adverse effects of homozygous deletion of *Vhl* upon β-cell function [230-233]. In these models, there was a massive increase in HIF-1α protein. Disruption of *Vhl* was accompanied by adverse gene expression changes, increased lactate, and severely impaired GSIS. These findings are interesting but were unexpected as heterozygous whole-body *Vhl* knockout mice appeared grossly normal [329], with 5 % – 25 % of mice developing abnormal vascular lesions in later life on some genetic backgrounds [330]. People with VHL syndrome may develop

endocrine pancreatic tumours, pancreatic cysts, and occasional insulinomas [300, 331-333]. However, less than 3 % of patients are reported to have abnormal glucose tolerance, despite frequently requiring steroids and/or pancreatic surgery [300, 331, 332, 334]. This suggests that a heterozygous germline mutation, as occurs in VHL patients, may not cause an increased risk of diabetes. Mutations in subunits of the succinate dehydrogenase complex [335, 336] and the HIF-1α prolyl hydroxylases are also associated with increased HIF-1α protein, but there are no reported alterations in diabetes incidence.

Loss of VHL was associated with decreased *Glut2* mRNA [230, 232]. We also found decreased *Glut2* with DFO at 16-times the therapeutic dose, exposure to 1 % oxygen [260], high dose *Vhl* siRNA, and supplemental iron. In contrast, therapeutic levels of DFO and 5 % oxygen treatment both caused different changes in gene expression, and in particular, *Glut2* was increased [260]. The different changes in gene expression with different methods of increasing HIF-1α were in accordance with the changes in β-cell function (Figure 4.27).

Factor inhibiting HIF (FIH) is an additional regulator of HIF-1α under normoxic conditions. Mouse with a knockout of *Fih* (*Fih*$^{-/-}$) were more insulin sensitive and had decreased weight gain on a HFD [235]. In addition, these mice had increased intracellular ATP levels accompanied with increased expression of *Pgk* and *Glut1*, but glycolysis was not observed to be changed [235]. The *Fih*$^{-/-}$ mice would reinforce the notion that increasing HIF-1α is not always deleterious, and it depends very much on the manner and how much HIF-1α is increased.

Thus, there appears to be a dose-response curve for HIF-1α (Figure 4.27). Deletion is deleterious in C57BL/6 mice and MIN6 cells. Mild increases are beneficial for β-cell function and glucose tolerance but very high levels, such as those achieved

with homozygous *Vhl* deletion, severe hypoxia, or overexpression of a degradation resistant mutant, are clearly deleterious for β-cell function.

Hydroxylation and proteolysis of HIF-1α requires iron, which is chelated by DFO and DFS. Iron overload due to transfusion dependency or haemochromatosis can cause β-cell dysfunction and increases diabetes incidence [337, 338]. It is perhaps less widely recognised that in the absence of transfusion-dependent iron overload or haemochromatosis, increases in serum ferritin or transferring saturation are associated with increased risk of diabetes and the metabolic syndrome [339-344]. High dietary iron intake is also associated with diabetes [341, 345]. Conversely, venesection and blood donation can improve β-cell function in people with diabetes [340, 346]. Regular blood donation has been reported to protect against diabetes, as does a vegetarian diet [337, 340, 346]. Disruption of the HIF-1α partner ARNT in endothelial cells leads to pronounced iron accumulation in the liver [347], suggesting the intriguing potential for a vicious cycle of decreased HIF-1α, decreased ARNT, increased iron accumulation, and decreased HIF-1α. Based on our data and the absence of a DFS effect in mice lacking β-cell HIF-1α, we postulate that a decrease in HIF-1α may be a mechanism contributing to the increased risk of diabetes with increased iron.

4.4.1 Conclusion

In conclusion, the combined use of mouse and tissue culture models utilised in this project have identified the following:

i) Both the β-*Hif-1α* mouse model and knockdown of *Hif-1α* in MIN6 cells have impaired first phase GSIS.

ii) Loss of *Hif-1α* in the β-cell and knockdown of *Hif-1α in* MIN6 cells reduced intracellular ATP and leads to reduced expression of key genes involved in glucose metabolism including *Glut2*, *Gck*, *Pfk*, and *Akt2*.

iii) Increasing HIF-1α protein by using a mutant form of HIF-1α or knocking down *Vhl* leads to impairments in GSIS.

iv) Stabilising HIF-1α by using the iron chelator DFS improves glucose tolerance in mice on a HFD and this was mediated by HIF-1α.

In summary, these studies demonstrate that β-cell HIF-1α is important for β cell reserve, and increasing HIF-1α by iron chelation markedly improved glucose tolerance on a HFD. Increasing HIF-1α normalised gene expression in T2D islets [260]. Therefore, we propose that increasing HIF-1α by iron chelation may be a valid therapeutic strategy for the treatment of human T2D.

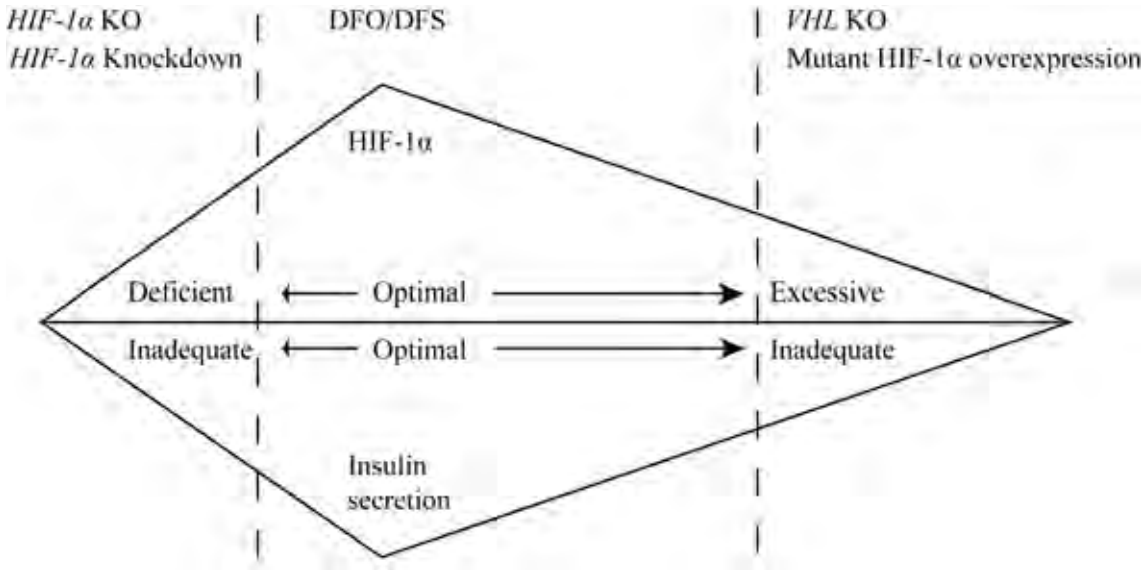

Figure 4.27 Model of HIF-1α required for optimal insulin secretion. We postulate that deficient β-cells deficient in HIF-1α leads to inadequate insulin secretion. However, excessive amounts of HIF-1α will also lead to impaired insulin secretion. We hypothesise that increasing HIF-1α by using the iron chelator DFO or DFS leads to optimal insulin secretion capacity.

Chapter 5

General discussion

5.1 Introduction

The experiments outlined in this thesis were designed to identify factors that regulate first phase insulin secretion. As detailed in Chapter 1, first phase insulin secretion is particularly important in the regulation of glucose homeostasis as it has a large role on the liver, suppressing EGP, and also primes insulin sensitive tissues to more readily take up glucose. A failure to reduce EGP can lead to IGT and loss of first phase insulin secretion is one of the earliest detectable predictors of T2D onset. Restoration of first phase could be an important first step in the treatment of T2D. Patients who have bariatric surgery rapidly regain first phase insulin secretion and glucose tolerance, sometimes prior to substantial weight loss.

Insulin secretion is a complicated system that involves a number of processes that involve the sensing and metabolism of glucose to provide the necessary signals for insulin release. Due to its complexity, there is still much that is not known regarding the regulation of insulin secretion and many studies have been performed in an attempt to understand this crucial phase of glucose homeostasis. Many of these regulatory factors have been outlined in Chapter 1.

These studies have examined two models of reduced first phase insulin secretion; high passage MIN6 cells and β–cells lacking *Hif-1α*.

5.2 High passage MIN6 cells

The MIN6 cell model for insulin secretion is used widely in research laboratories as an *in vitro* model for insulin secretion. It is known that high passage MIN6 cells lose their ability secrete insulin in response to glucose stimulation, however this is the first reported study to identify that high passage MIN6 cells lost first phase but still retain partial second phase GSIS. This is an important distinction in that loss of first phase insulin secretion is one of the first identifiable indicators of T2D onset and people with

T2D often continue to have normal or enhanced second phase insulin secretion for years after diagnosis. Identifying factors that cause loss of first phase insulin secretion in high passage MIN6 cells could be important in developing treatments for T2D patients.

These studies have identified a number of metabolic changes that occurred in high passage MIN6 cells that contributed to loss of first phase insulin secretion. We believe that the changes that occurred, including increased basal glucose oxidation and uptake, and decreased basal lipid oxidation were part of a compensatory system in a failed attempt to increase intracellular ATP, an important factor for insulin secretion which high passage MIN6 cells were clearly lacking. Islets from T2D patients have reduced ATP when stimulated with glucose, ultimately leading to a reduced capacity to secrete insulin [348]. In addition to this, islets from T2D patients also have reduced glucose oxidation when stimulated with glucose, much like that observed in high passage MIN6 cells [74]. Although the decreases were not of the same magnitude as that seen in the high passage MIN6 cells, we may still be able to draw some comparisons between metabolic changes in high passage MIN6 cells and T2D islets.

5.2.1 High passage MIN6 cells as a model of reduced first phase insulin secretion

The best and most physiologically relevant model for reduced first phase insulin secretion would be islets from a T2D patient. However, these islets are hard to obtain due to obvious reasons. Animal such as mice are the next logical model and many already exist in diabetes research. These include db/db, ob/ob, and the New Zealand Obese (NZO) mice just to name a few. However, animals too have their disadvantages:

- Animal models require dedicated facilities and staff for their upkeep and thus cost is increased.
- Animals need time to grow and reach an age where experiments can be performed

- Variances can also arise from animal models, especially with age and sex, adding a degree of difficulty in interpretation of results.
- It can be difficult to distinguish between direct effects on β-cell function and indirect effects via other tissues in whole animal systems.

Cell culture offers a cost effective and time efficient alternative to animal and human models and can provide man useful insights. In the case of this project, we describe a model whereby cells lose first phase but still retain partial second phase GSIS. This phenotype is very similar to that observed in humans in the early stages of T2D onset. High passage MIN6 cells offer a nearly unlimited supply of cells in which molecular phenotyping can be performed.

5.3 The role of HIF-1α in β-cell function

As mentioned in Section 1.9.1, a study by Gunton and colleagues [172] established quite clearly that both *ARNT* and *HIF-1α* expression were decreased in T2D islets and mice lacking *Arnt* in the β-cells had impaired glucose tolerance and insulin secretion. As ARNT binds with HIF-1α to form the heterodimer HIF-1, it begged the question of whether decreasing HIF-1α in β-cells would also worsen glucose tolerance. Indeed, this hypothesis proved to be correct and deletion of HIF-1α in the β-cells of mice caused impaired glucose tolerance and abolished first phase insulin secretion. We confirmed these results by knocking down HIF-1α in MIN6 cells and this also reduced GSIS.

Interestingly, while this project was underway, four independent groups published results clearly demonstrating that mice lacking *Vhl* in the pancreas and/or β-cells, and thus increasing HIF-1α protein stability, were detrimental to glucose tolerance and insulin secretion [230, 232, 233]. This raises an interesting scenario in which both deletion of *Hif-1α* and increasing HIF-1α protein (by deleting *Vhl*) in mice both impaired insulin secretion. This suggests a “Goldilocks” paradigm analogous to the

effect of ROS, where too much and too little are both deleterious for β-cell function [158, 160]. Interestingly, HIF-1α is both regulated by and regulates ROS [349-351]. Not enough HIF-1α is detrimental [230-233]. However, increasing HIF-1α protein by using an iron chelator to the point where it is "just right", and not to the levels observed by deleting *Vhl*, is beneficial for glucose tolerance.

This is supported by a study by Zhang et al investigating the effects of knocking out factor inhibiting HIF-1α (FIH) [235]. Under normoxic conditions, HIF-1α protein is highly regulated at the protein level by hydroxylases, with FIH being one of them. Mice with *Fih* knocked out displayed none of the characteristics of a *Vhl* knockout, instead these mice had improved insulin sensitivity and decreased weight, even under a high fat diet challenge [235]. These results are similar to those reported by our group using iron chelation to stabilise HIF-1α protein activity [260]. Additional circumstantial support is provided by the fact that people with VHL syndrome in whom there are heterozygous mutations in VHL appear to be protected from diabetes development, especially after pancreatic resections [300, 331, 332, 334]. To our knowledge, glucose tolerance in mice with heterozygous *Vhl* defects in the pancreas has not been reported, and would be of particular interest.

5.3.1 Iron stores and diabetes

There is no doubt that iron is required for many metabolic functions and one of the major users of iron is the mitochondrion. However, iron is also a major inducer of ROS production and thus iron homeostasis needs to be maintained within the mitochondrion [352-354]. The inability of mitochondria to maintain iron homeostasis, thus increasing ROS production, can lead to a number of diseases, including Friedriech's ataxia [355], sideroblastic-like microcytic anaemia [356], and myopathy [357]. As normal mitochondrial function is required for insulin secretion, it may be possible that increases

in mitochondrial iron can lead to impaired glucose homeostasis caused by ROS induced mitochondrial dysfunction.

An association between iron status and diabetes has been suggested previously. Many studies have shown that increased iron stores are positively correlated with risk of T2D [340, 343, 358, 359] and people on a vegetarian diet have been observed to have a lower risk of T2D due to many factors including reduced fat intake, weight gain, and iron stores [360-362]. Some studies have shown that reducing iron stores by other means such as frequent blood donations or phlebotomy are associated with reduced risk of T2D and improving the symptoms of T2D patients with high iron [337, 340, 346], while others have argued that there is no correlation at all [341]. Investigating methods to reduce iron stores may be of some benefit to T2D patients based on prior evidence.

The results of this study raise the notion of HIF-1α acting as a nutrient sensor. It is understood that HIF-1α acts as the oxygen sensor and this is due to the actions of the hydroxylases as both of them require oxygen to function. In addition to this, both hydroxylases also require iron to function (as discussed in Section 1.9.2) and if we extrapolate upon this requirement, HIF-1α could act as a potential nutrient sensor. When dietary intake of iron is low, such as in vegetarian diets, a decrease in hydroxylase function would allow HIF-1α protein to accumulate and improve insulin secretion. The reverse of this would be that diets high in iron, in addition to increasing ROS, would inhibit HIF-1α activity and glucose homeostasis. It is unknown whether there are any changes in HIF-1α protein in people on a vegetarian diet and whether it has any correlation to their lowered risk of T2D but this would be interesting to determine.

5.3.2 DFO and DFS as a treatment for T1D and T2D

The use of iron chelators have been used previously in the treatment of a variety of diseases associated with high iron levels. Iron chelation therapy has been shown to reduce proliferation of vascular smooth muscle cells in patients with atherosclerosis [363] and improved conditions for patients with thalassemia major [364]. Deferoxamine (DFO) is the parenteral analogue of DFS and has been previously investigated in the setting of both T1D and T2D models. Roza and colleagues [365] showed that hydroxyethyl starch DFO reduced the incidence of disease in rats that spontaneously develop diabetes. More recently, DFO was shown to improve the function of encapsulated human islets, possibly via the actions of increased vascular endothelial growth factor (VEGF) and HIF-1α [366]. A submitted paper from our lab indicates that DFO was able to improve human islet function transplanted into mice, with increased vascularisation and decreased apoptosis [367].

With respect to T2D, a number of recent studies have shown improvements in disease conditions in animal models using iron chelation or iron restriction therapy. The use of iron chelators and iron restriction diets were used successfully in the *ob/ob* leptin deficient mice and the Otsuka Long-Evans Tokushima fatty rats in protecting against and reducing the severity of T2D [368, 369]. In addition to this, Tajima and colleagues [370] showed that the use of DFO reduced oxidative stress leading to improved glucose tolerance and insulin sensitivity, while reducing adiposity in the obese T2D KKAy mice. However, the effects of iron chelation therapy are wide reaching as iron is required for a number of cellular functions and the exact pathway(s) DFO is acting through is yet to be understood.

The experiments performed in this project would indicate that DFS is acting via the functions of HIF-1α. When control and β-*Hif-1α* mice were placed on a HFD with DFS, only the control mice showed improved glucose tolerance, showing that β-cell

HIF-1α was absolutely required for the benefit in glucose tolerance. The experiments performed in this project were in C57BL/6 mice but similar improvements were seen in Balb/c mice when given a HFD with DFS [260]. Therefore, the beneficial effects of DFS observed in the control mice were acting directly via the HIF-1α pathway.

5.3.2.1 Efficacy of DFO and DFS

The iron chelator DFS is already approved for human use in the treatment of iron overload and the concentrations used in this study were within the human therapeutic dose range. Long term studies in this laboratory have found no adverse effects after treatment with DFS for up to 35 weeks [371]. In humans, early studies of the parental form DFO found some subjects displayed visual and auditory neurotoxicity [372, 373] however the overall risk to benefit ratio of using this drug is generally acceptable. Side effects of the oral from DFS include abdominal pain, nausea, diarrhoea, and skin rash [374]. There are rare reports of agranulocytosis in people with bone marrow dysfunction, and an idiosyncratic renal impairment which usually resolves with cessation or dose reduction [375]. These incidences seem to be rare and most reports have found DFS usage within the recommended doses have beneficial effects for patients [376, 377]. However, as in the case for most drugs, continued monitoring in patients and research is always advised.

5.4 Future directions

5.4.1 MIN6 cells

Unfortunately, a unifying single mechanism for reduced first phase insulin secretion was not identified in high passage MIN6 cells. Many interesting gene expression changes were observed in high passage MIN6 cells that would be of interest to follow up. Members of the sirtuin family and *Nampt* have been previously associated with

reduced insulin secretion and both *Sirt3* and *Nampt* expression were decreased in high passage MIN6 cells. Gene expression data alone cannot positively implicate these genes as the cause of reduced first phase insulin secretion in high passage MIN6 cells and thus it would be necessary to determine if protein expression is also reduced to reflect gene expression changes. Next, it would of great interest to ascertain what the functions of these genes have in MIN6 cells, if any. This could be achieved by using siRNA to knock down *Sirt3* and/or *Nampt* expression in low passage MIN6 cells and observe any defects in GSIS and glucose metabolism. If knocking down *Sirt3* and/or *Nampt* is phenotypically similar to high passage MIN6 cells then this would provide a strong case for its role.

Many functional changes were identified in high passage MIN6 cells, some of which we know to occur in T2D patients. A previous study has shown that T2D islets have reduced glucose oxidation when stimulated with glucose [74], increased whole body glucose uptake at basal glucose levels [287], and impaired insulin response to L-arginine [286]. Islets from T2D patients also had reduced ATP content when stimulated with glucose [348] and this was similar to that seen in high passage MIN6 cells. The authors argued that reduced ATP content in T2D islets were due to observed increases in UCP2, acting in this case, as a mitochondrial uncoupler. Although UCP2 expression in high passage MIN6 cells was decreased compared to low passage MIN6 cells, the uncoupling actions of UCP2 is contentious, as discussed in Section 4.4. As endogenous ATP content is lowered in high passage MIN6 cells, an interesting question to answer is will the addition of exogenous ATP aid in GSIS?

Lipid oxidation can provide much needed energy for insulin secretion and while this was observed to be impaired in high passage MIN6 cells, little is known in T2D islets. Increased lipid oxidation in muscle has been associated with insulin resistance [146] but whether it is altered in T2D islets would be interesting to determine. Basal

lipid oxidation was increased in high passage MIN6 cells and forcibly reducing this by using bromopalmitate markedly and rapidly increased cell death. Retinoic acid has been used to increase lipid oxidation in mice [378] but whether or not this would work in a cell culture model remains to be seen.

There were also obvious limitations in the experiments performed in this study. It would be extremely beneficial to perform a perifusion assay to accurately measure first and second phase insulin secretion in high and low passage MIN6 cells. It would be unlikely that this would alter the outcomes, but the static insulin secretion assays as performed in this study cannot achieve the accuracy of perifusion assays. It would also be helpful to employ more advanced technology, such as Total Internal Reflection Fluorescence microscopy to analyse how insulin granules are being secreted in high and low passage MIN6 cells to determine if any changes are present.

5.4.2 HIF-1α

The experiments in this project have identified HIF-1α as an important regulator of glucose homeostasis and first phase insulin secretion, possibly due to its regulation of many key glycolytic genes. Animal and cell culture models lacking HIF-1α show that deletion is detrimental to glucose homeostasis. Conversely, increasing HIF-1α using the iron chelator DFS improved glucose tolerance in mice on a HFD. Therefore, we have identified a possible new drug treatment for patients with T2D, one that is already approved for human use in iron overload.

Unpublished data from experiments performed in our laboratory have examined metabolic changes that occur in mice fed DFS to determine underlying mechanisms [371]. Although we have gained a little more understanding into the actions of DFS and how it may function, there is still more to be done. Our mice did not have any observed adverse effects when treated with DFS for up to 35 weeks [371]. However, as stated

above, there have been some reports of visual and auditory neurotoxicity and it will be necessary to determine if mice treated long term with DFS have any of these effects.

Mice treated with DFS for 26 weeks gained less weight that the control mice on a HFD [260]. However, it would be interesting to determine what would happen if DFS was removed in the treated group? Would the mice continue to gain weight slower? How rapidly would the benefit wear off? The experiments in this study have shown an improved in glucose tolerance after three weeks on a HFD but would this still occur if the mice were on a HFD for longer before treatment? These are questions that would be of interest in further studies.

5.5 Conclusion

The experiments in this project have examined two models of reduced first phase insulin secretion: high passage MIN6 cells and mice lacking HIF-1α in the β-cells. In addition to this, we have identified a possible new therapeutic drug for the treatment of T2D. The results have given us a greater understanding of the metabolic changes that produce defective, and also an important regulator of first phase insulin secretion. As first phase insulin secretion is crucial to glucose homeostasis and loss of it invariably leads to IGT and T2D, the results in this study will only aid in better treatment options for T2D patients.

Chapter 6

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Chapter 7

Publications arising from this thesis

OPEN ACCESS Freely available online

PLoS one

High Passage MIN6 Cells Have Impaired Insulin Secretion with Impaired Glucose and Lipid Oxidation

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Abstract

Type 2 diabetes is a metabolic disorder characterized by the inability of beta-cells to secrete enough insulin to maintain glucose homeostasis. MIN6 cells secrete insulin in response to glucose and other secretagogues, but high passage (HP) MIN6 cells lose their ability to secrete insulin in response to glucose. We hypothesized that metabolism of glucose and lipids were defective in HP MIN6 cells causing impaired glucose stimulated insulin secretion (GSIS). HP MIN6 cells had no first phase and impaired second phase GSIS indicative of global functional impairment. This was coupled with a markedly reduced ATP content at basal and glucose stimulated states. Glucose uptake and oxidation were higher at basal glucose but ATP content failed to increase with glucose. HP MIN6 cells had decreased basal lipid oxidation. This was accompanied by reduced expressions of *Glut1, Gck, Pfk, Srebp1c, Ucp2, Sirt3, Nampt*. MIN6 cells represent an important model of beta cells which, as passage numbers increased lost first phase but retained partial second phase GSIS, similar to patients early in type 2 diabetes onset. We believe a number of gene expression changes occurred to produce this defect, with emphasis on *Sirt3* and *Nampt*, two genes that have been implicated in maintenance of glucose homeostasis.

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Introduction

Type 2 diabetes (T2D) is characterized by the inability of beta-cells to secrete enough insulin to maintain glucose homeostasis, usually accompanied by insulin resistance: impaired action of insulin on target tissues. Beta-cells secrete insulin in a biphasic manner. The first phase occurs rapidly, commonly defined as <10 minutes after glucose stimulation. In the whole body, insulin secretion normally peaks at 1–2 minutes [1]. Second phase secretion is more gradual and long lasting, typically reaching a plateau at 25–30 minutes [1].

The classic pathway of insulin secretion is generally accepted and begins with glucose entry into beta-cells, and a rise in the ATP:ADP ratio as glucose is metabolized via glycolysis and oxidative phosphorylation [2–4]. This closes ATP-dependent K^+ channels (K_{ATP}) and depolarizes the plasma membrane [5,6]. This then opens the voltage-gated calcium channels, allowing Ca^{2+} to enter the cells, leading to subsequent insulin exocytosis [7]. Interestingly, patients early in the course of T2D lose first phase but often retain second phase insulin secretion [8]. Impairment of first phase secretion is a predictor of future type 1 and T2D risk [9]. Many additional mechanisms regulate insulin secretion including the rate of anapleurosis and changes in glutamate, GTP, and GDP levels (all of which are involved in the amplifying pathway) [4].

The MIN6 cell line was derived from a mouse insulinoma and is one of a few cell lines that display characteristics of pancreatic beta-cells, including insulin secretion in response to glucose and other secretagogues [10,11]. It has been reported that MIN6 cells with high passage (HP) numbers lose their ability to secrete insulin [12–14]. HP MIN6 cells have gene expression changes, including downregulation of genes such as *phospholipase D1* and *cholecystokinin* [14].

HP and low passage (LP) MIN6 cells also differ at the protein level, with HP cells having lowered expression of some proteins that are involved with correct protein folding in the ER and antioxidant enzymes for handling of ROS [12]. This is in contrast to studies comparing glucose responsive and unresponsive sublines of LP MIN6 cells, with one study showing no change in Glut2 expression [15] while another study showing barely detectable Glut2 expression, even in their glucose responsive MIN6 cell line [16].

These studies showed changes in gene and protein expression, however metabolic changes have not been profiled with a panel of functional assays in detail before. We sought to identify changes associated with impaired insulin secretion in HP cells. HP cells lost first phase insulin secretion and had an overall impairment in GSIS. This was coupled with a markedly reduced intracellular ATP in HP cells, with decreased glucose uptake, glucose oxidation and basal lipid oxidation. HP MIN6 cells had significantly reduced expression of glycolytic genes and genes involved with lipid

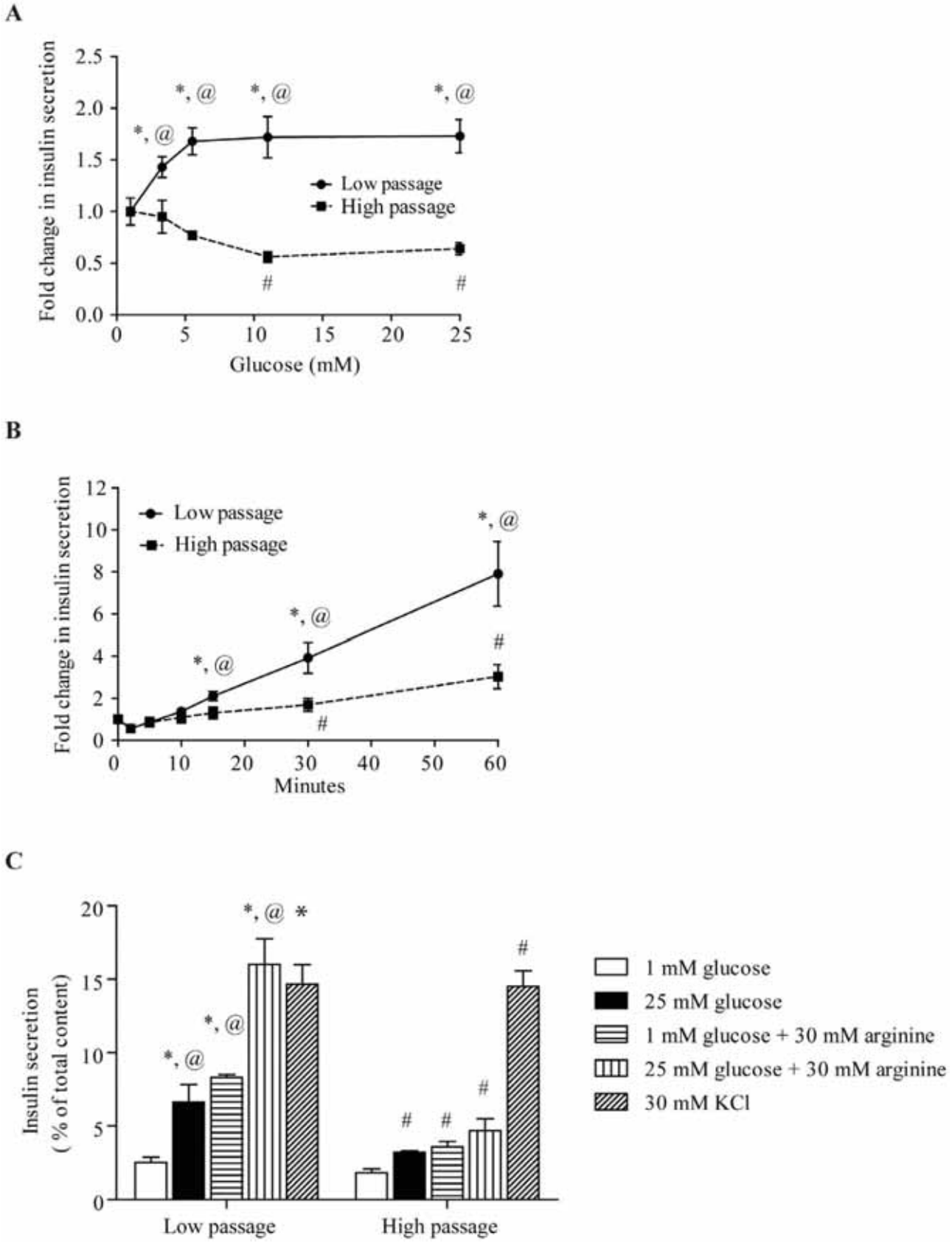
A
Fold change in insulin secretion
2.5
2.0
1.5
1.0
0.5
0.0
*, @
*, @
*, @
*, @
Low passage
High passage
#
#
0
5
10
15
20
25
Glucose (mM)
B
Fold change in insulin secretion
12
10
8
6
4
2
0
Low passage
High passage
*, @
*, @
*, @
#
#
0
10
20
30
40
50
60
Minutes
C
Insulin secretion
(% of total content)
20
15
10
5
0
*, @
*, @
*, @ *
#
#
#
#
Low passage
High passage
1 mM glucose
25 mM glucose
1 mM glucose + 30 mM arginine
25 mM glucose + 30 mM arginine
30 mM KCl

Figure 1. Insulin secretion in LP and HP MIN6 cells. A) LP MIN6 cells responded in a dose dependent manner to glucose but this was not observed in HP MIN6 cells (samples taken at 15 minutes after stimulation, representing first phase insulin secretion). B) A GSIS time course showed that HP MIN6 cells still retained partial second phase insulin secretion, although significantly lower than that observed in LP MIN6 cells. C) After an incubation time of 30 minutes, LP MIN6 cells responded well to the non glucose secretagogues L-arginine and KCl. HP MIN6 cells showed no augmentation in insulin secretion with L-arginine but responded to KCl, with levels comparable to LP MIN6 cells. Error bars are ± SEM and n = 6. * $p<0.05$ compared to LP MIN6 cells at basal, # $p<0.05$ compared to HP MIN6 cells at basal, @ $p<0.05$ LP vs. HP MIN6 cells at their respective glucose concentrations or time points.
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handling, including *Srebp1c*. This was accompanied by a decrease in *Sirt3* and *Nampt* gene expression.

Materials and Methods

Cell Culture

We obtained MIN6 cells from Dr. Ross Laybutt (Garvan Institute of Medical Research) [17] (originally from Dr Miyazaki [11]) and cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 25 mM Hepes, and 285 μM 2-mercaptoethanol. Subculture and maintenance were performed as previously described [13]. MIN6 cells presented in this study were at passages 30–40 (low passage, LP) or passages 60–70 (high passage, HP). We compared earlier passage cells (P26–27) and they did not differ in normal GSIS from P30–40 (data not shown). All assays used MIN6 grown to 70–80% confluence unless otherwise stated.

Electron Microscopy

Electron microscopy samples were fixed for 30 minutes in cacodylate buffered 2% glutaraldehyde. Samples were post-fixed for 90 minutes in 2% osmium tetroxide and then enbloc stained with 2% aqueous uranyl acetate (30 minutes). Samples were dehydrated through a series of ethanols and infiltrated with TAAB epoxy resin. The blocks were set at 80°C overnight. Methylene blue/Azure II stained thick sections were used to identify suitable areas of the blocks which were cut at 100 nm. These sections were collected on Cu/Pd grids, stained with Reynolds lead citrate and viewed on a JEOL 1011 electron microscope with images captured using a MegaView III digital camera and AnalySIS software package.

Cell Proliferation

Cell proliferation was examined using the FITC BrdU Flow Kit (BD Pharmingen, San Diego, CA, USA). MIN6 cells were pulsed with 10 μl/ml of BrdU solution (1 mM BrdU in PBS) for 40 minutes in MIN6 media at 37°C with 5% CO_2. Cells were dislodged by trypsin. BrdU and 7AAD staining was performed as per the protocol. Flow cytometric data was acquired using a FACS Calibur (BD Biosciences, San Jose, CA, USA) and FlowJo software (Tree Star).

Insulin Secretion Assays

Media used for insulin secretion was serum free DMEM with no glucose, supplemented with 2 mM L-glutamine and 25 mM Hepes, pH 7.4. Glucose was added to prepare basal (1 mM) and other glucose concentrations (3.3 mM, 5.5 mM, 11 mM, and 25 mM) and warmed to 37°C prior to use. MIN6 cells were washed twice with PBS and placed in basal serum free media for 2 hours, washed with fresh basal media and placed in the stated media for 15 minutes. Media was then replaced with media containing higher concentrations of glucose for 15 minutes. 15 minutes was the minimum time necessary for the cells and culture media to reach 37°C (approx. 5 minutes) and insulin secretion (approx. 10 minutes) to occur. After completion of the incubations, the cells were lysed with acid/ethanol for measurement of total insulin content as previously reported [18,19]. Insulin secretion with addition of 1 mM pyruvate, 30 mM L-arginine, or 30 mM KCl was performed after an incubation time of 30 minutes. Bromopalmitate (0.0625 μM) was added 2 hours before insulin secretion assays.

For insulin secretion time courses, MIN6 cells were grown in 12-well plates and samples for basal 1 mM insulin secretion was collected as above. After the addition of 25 mM serum free media, 10 μl was removed at each time point (2, 5, 10, 15, 30, 60 minutes) and total insulin collected as described above. Insulin was measured by ELISA (Crystal Chem, Downers Grove, IL, USA).

Measurement of Intracellular ATP Content

Intracellular ATP content was measured using the ATP Bioluminescence Assay Kit CLS II (Roche, Sydney, NSW, Australia) according to the manufacturer's instructions. Cells were equilibrated as described above then serum free media was replaced with fresh basal media. Glucose was added to make up to 25 mM at each time point. MIN6 cells were placed on ice, washed twice with ice cold PBS, and lysed. Results were corrected for total protein.

Glucose Oxidation Assay

MIN6 cells were grown in 25 cm^2 flasks, washed twice with PBS, and incubated in Krebs buffer (115 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1 mM sodium pyruvate, 10 mM Hepes, pH 7.4) with 1mM glucose for 2 hours. After equilibration, cells were washed with fresh Krebs buffer with 1mM glucose and media replaced with fresh Krebs buffer at 1, 5, 11, or 25 mM glucose with 0.1 μCi/ml of D-[U-^{14}C]-glucose (GE Healthcare, Port Washington, NY, USA). Filter paper soaked in 5% KOH was suspended over the cells and the flasks sealed shut. Cells were incubated at 37°C with 5% CO_2 for 1 hour and the reaction stopped by the addition of perchloric acid. Radioactivity was counted in 4 ml Microscint-20 (Perkin Elmer, Waltham, Massachusetts, USA) using the LS 6500 Scintillation Counter (Beckman Coulter, Brea, CA, USA). Results were corrected for specific activity and total protein.

Glucose Uptake Assay

MIN6 cells were grown in 6-well plates and equilibrated as per glucose oxidation protocols. After washing, media was replaced with fresh warm Krebs buffer with 1 or 25 mM glucose and 1 μCi/ml 2-deoxy-[1,2-^{3}H]-glucose (Perkin Elmer, Waltham, Massachusetts, USA). Cells were incubated at 37°C with 5% CO_2 for exactly 5 minutes. The reaction was stopped by placing on ice and washing twice with ice cold 5% glucose in PBS. Cells were lysed with 500 μl of RIPA buffer (0.5% sodium deoxycholate, 50 mM Hepes, 1% NP40, 0.1% SDS, pH 7.4) and counted as above. Results were corrected for total protein.

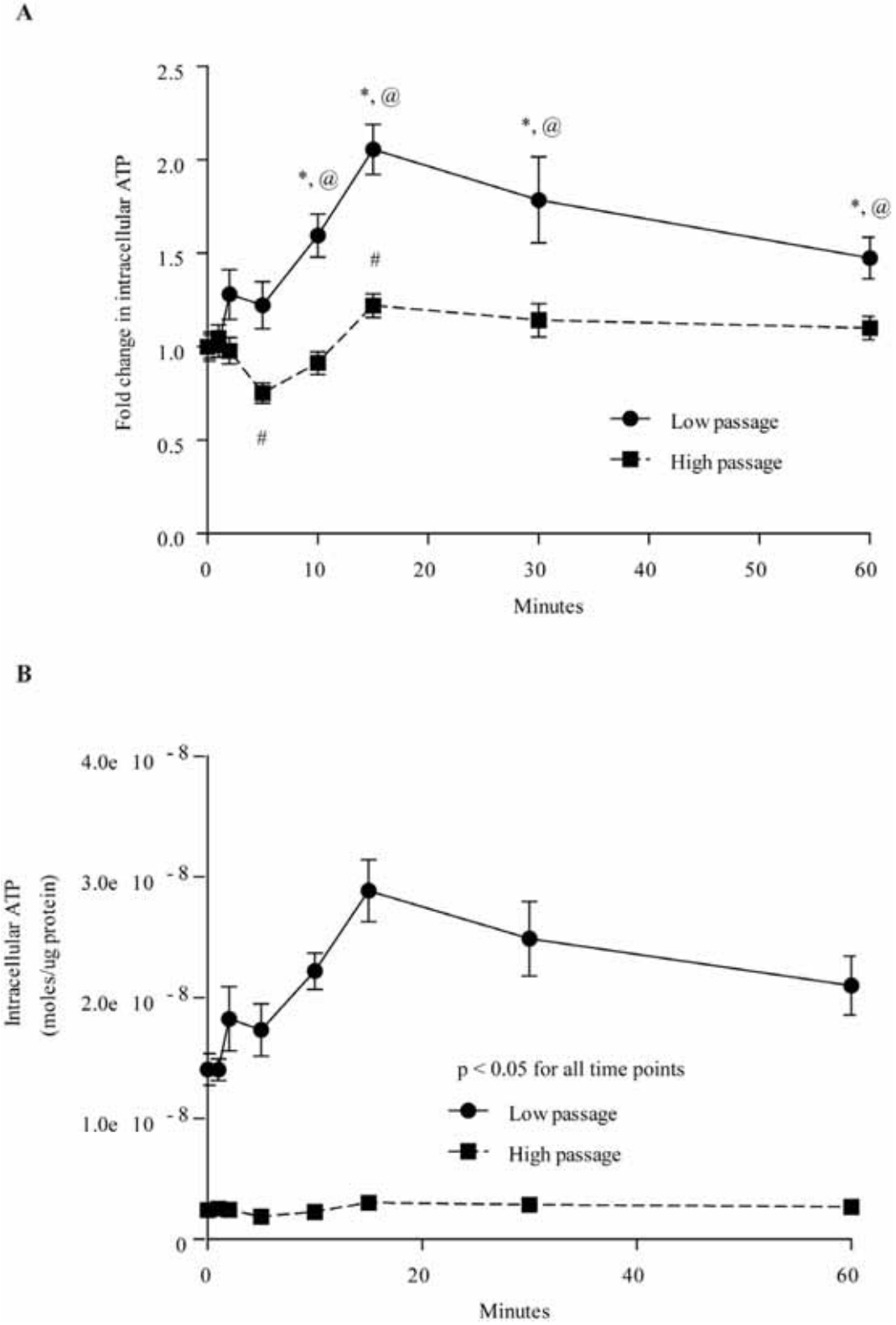

Figure 2. Intracellular ATP content in LP and HP MIN6 cells. A) Time course showing the fold change in intracellular ATP content. HP MIN6 cells only exhibited significant fold change differences from basal ATP content at 2 and 15 minutes after glucose stimulation. LP MIN6 cells had significantly higher fold changes from basal after 10 minutes of glucose stimulation. B) Time course showing the intracellular ATP molar concentration. HP MIN6 cells had significantly less intracellular ATP content at all time points. Error bars are ± SEM and n = 6. * $p<0.05$ compared to LP MIN6 cells at basal, # $p<0.05$ compared to HP MIN6 cells at basal, @ $p<0.05$ LP vs. HP MIN6 cells at their respective time points.
doi:10.1371/journal.pone.0040868.g002

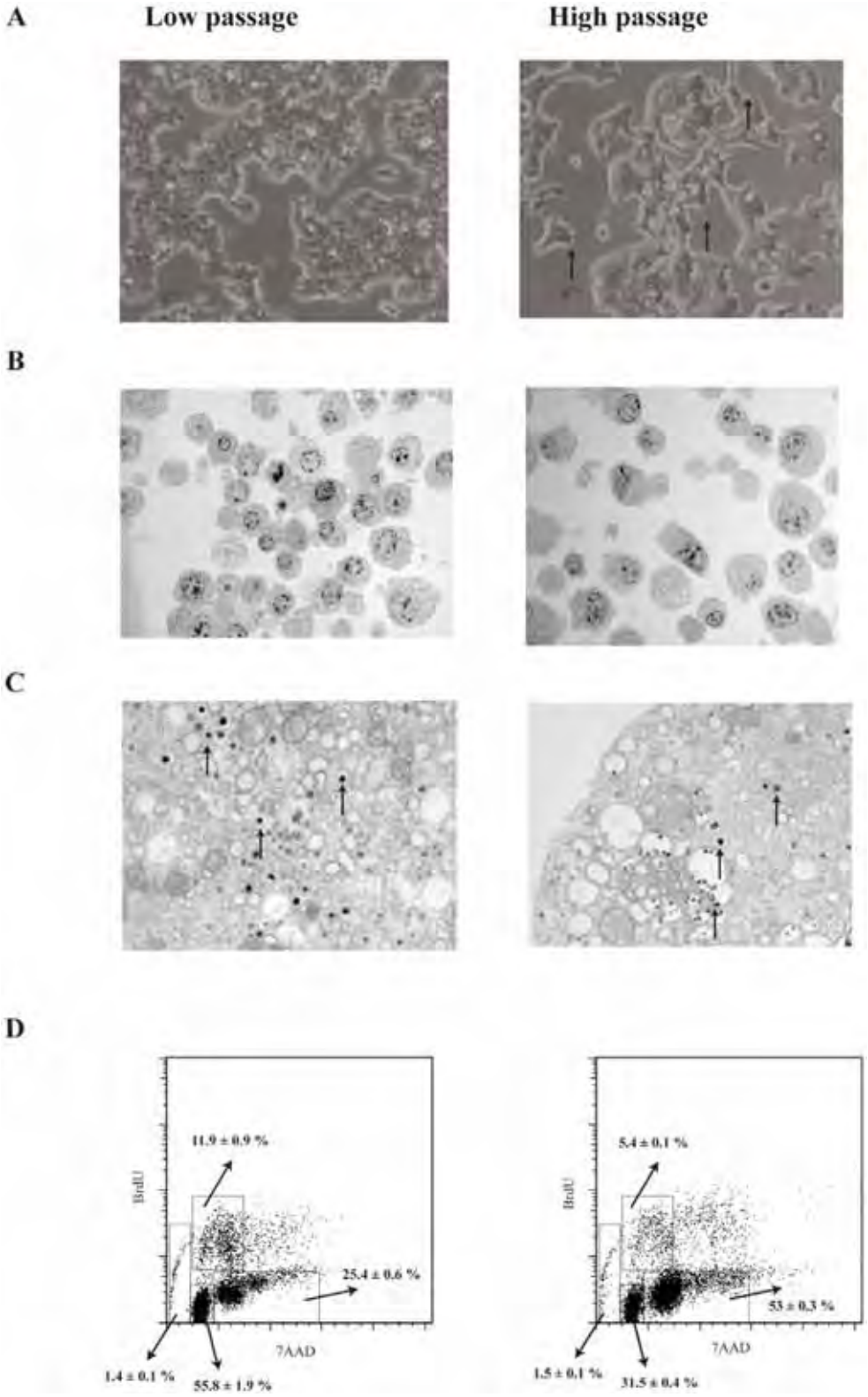

Figure 3. Morphology and cell proliferation in LP and HP MIN6 cells. A) Pointed formations (as indicated by the arrows) were observed in HP MIN6 cells and appeared more irregular than LP MIN6 cells. B) Electron microscope pictures of LP and HP MIN6 cells under low magnification. HP MIN6 cells were larger compared to LP MIN6 cells (200.56 μM^2 versus 111.89 μM^2, $p<0.05$). C) LP MIN6 cells displayed distribution and frequency of insulin granules (as indicated by the arrows) typical of an islet cell while HP MIN6 cells had slightly less insulin granules. D) Representative FACS plot of BrdU and 7AAD staining. HP MIN6 cells had a lower percentage of cells in the S phase of DNA replication (5.4% versus 11.9%, $p<0.05$). Errors values are $\pm$ SEM and $n=3$.
doi:10.1371/journal.pone.0040868.g003

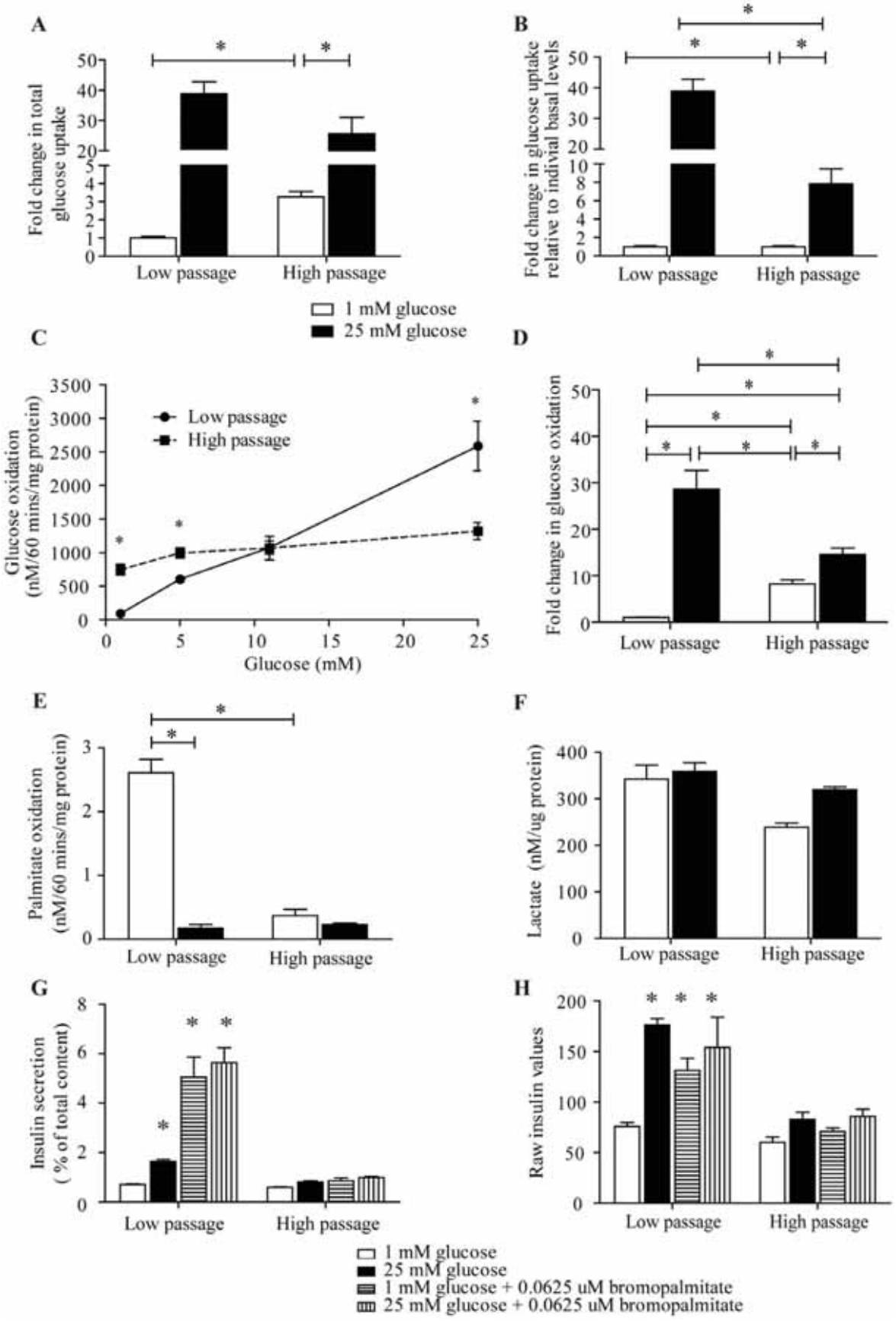
A
Fold change in total glucose uptake
Low passage
High passage
B
Fold change in glucose uptake relative to indivial basal levels
Low passage
High passage
1 mM glucose
25 mM glucose
C
Glucose oxidation (nM/60 mins/mg protein)
Low passage
High passage
Glucose (mM)
D
Fold change in glucose oxidation
Low passage
High passage
E
Palmitate oxidation (nM/60 mins/mg protein)
Low passage
High passage
F
Lactate (nM/ug protein)
Low passage
High passage
G
Insulin secretion (% of total content)
Low passage
High passage
H
Raw insulin values
Low passage
High passage
1 mM glucose
25 mM glucose
1 mM glucose + 0.0625 uM bromopalmitate
25 mM glucose + 0.0625 uM bromopalmitate

Figure 4. Glucose uptake, glucose oxidation, and lipid oxidation in LP and HP MIN6 cells. A) Both LP and HP MIN6 cells had a significant increase in total glucose uptake from 1 to 25 mM glucose when compared to 1 mM glucose uptake in LP cells. B) When fold change in glucose uptake is compared to their respective basal levels, HP cells only had an approximate 7-fold increase compared to an approximate 38-fold increase in LP cells. C) LP MIN6 cells exhibited a dose response in glucose oxidation when stimulated with increasing amounts of glucose. HP MIN6 cells did not show this response, with an 8-fold increase in glucose oxidation at 1 mM glucose and only increasing by 1.8-fold at 25 mM glucose. D) Glucose oxidation in low and high passage MIN6 cells expressed as the fold change compared to LP MIN6 cells at 1 mM glucose. E) HP MIN6 showed no preferential lipid oxidation at low glucose concentrations as that seen in LP MIN6 cells. There were no significant differences in lipid oxidation between 1 and 25 mM glucose in HP MIN6 cells. F) There was a significant increase in lactate from 1 to 25 mM glucose. G) 0.0625 μM bromopalmitate treatment for 2 hours would seem to increase insulin secretion but total insulin values content was low indicating cell death. H) Raw insulin values showing reduced insulin secretion with bromopalmitate treatment in both LP and HP MIN6 cells. Error bars are ± SEM and n = 6. * $p<0.05$.
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Lipid Oxidation

MIN6 cells were grown in 25 cm^2 flasks, washed twice with PBS, and incubated in Krebs buffer plus 0.25% fatty acid free BSA (Sigma-Aldrich, St. Louis, MO, USA) with 1 mM glucose at 37°C with 5% CO_2 for 2 hours. After equilibration, cells were washed with fresh Krebs+BSA buffer with 1 mM glucose and media replaced with fresh Krebs+BSA buffer at 1 or 25 mM glucose concentrations with 0.125 mM palmitate and 0.25 μCi/ml of [1-^{14}C]-palmitic acid (GE Healthcare, Port Washington, NY, USA). Filter paper soaked in 5% KOH was suspended over the cells and the flasks sealed shut. MIN6 cells were incubated at 37°C with 5% CO_2 for 24 hours and the reaction stopped by the addition of perchloric acid. Radioactivity was measured as above. Results were corrected for total protein.

Measurement of Lactate

Cells were incubated in serum free media containing 1 or 25 mM glucose for 2 hours and lactate was measured using the BioVision Lactate Assay Kit II (BioVision, Mountain View, Cal, USA) according to manufacturer's protocol. Results were corrected for total protein.

Real-time PCR

RNA was isolated as previously described [18] and real-time PCR performed as previously described [18,19]. Primer sequences are available on request. Every plate included a house-keeping gene (TATA-box binding protein (TBP)) for LP and HP cells.

Statistical Analysis

For all figures, error bars indicate ± SEM. Unpaired 2-tailed t-tests were used to compare two variables, and ANOVA with post-hoc testing (Bonferoni or Tukey's) was used for multiple comparisons. A p-value of <0.05 was considered significant.

Results

Insulin Secretion

Low passage (LP) was defined as passages 30–40 and high passage (HP) as passages 60–70. As shown in Figure 1A, LP cells displayed a dose response in insulin secretion when stimulated with increasing glucose concentrations. This culminated in a ~1.7-fold increase in insulin secretion with high glucose (25 mM) ($p<0.05$). This response was not observed in HP cells. There was a trend for higher insulin release at basal glucose (1 mM, $p = 0.2$, data not shown). Glucose stimulated insulin secretion time courses were studied and as expected, LP MIN6 cells secreted increasing amounts of insulin over time (Figure 1B). Interestingly, HP cells eventually responded to glucose at 60 minutes, however this was still significantly less than LP MIN6 cells ($p<0.05$). Thus, HP cells had an overall impairment in GSIS, with a more severe effect on first phase secretion.

Non-glucose secretagogues were next used. Potassium chloride (KCl) treatment at 30 mM induced strong and equivalent insulin secretion in both LP and HP cells, demonstrating that HP cells were still able to secrete insulin. KCl stimulated insulin secretion in HP was significantly greater than with 25 mM glucose ($p<0.05$, Figure 1C). L-arginine, is a potentiator of GSIS, was effective in both cells ($p<0.05$, Figure 1C) but HP cells did not achieve levels near those in LP cells or with KCl stimulation.

Intracellular ATP Content in Low and High Passage MIN6 Cells

LP cells responded to 25 mM glucose with increased ATP content, resulting in significant increases over basal levels from 10 minutes onwards ($p<0.05$, Figure 2A). This was not observed in HP cells, with the only significant increase at 15 minutes (Figure 2A). Figure 2A shows the intracellular ATP content expressed as a fold change compared with their respective basal levels. Surprisingly, there was a significant decrease in ATP content at 5 minutes in HP cells, which was not seen in LP cells ($p<0.05$). Figure 2B shows the ATP data expressed as raw numbers. LP cells had at least 5-fold higher ATP at all time points compared to HP cells ($p<0.01$).

Morphology and Cell Cycle Progression

Morphologically, LP MIN6 cells generally appeared round whereas the HP cells had a more irregular shape with pointed protrusions (Figure 3A). Cells were viewed under an electron microscope. HP cells were larger with an average size of 200.56 μM^2 compared to an average size of 111.89 μM^2 in LP MIN6 cells ($p<0.05$, Figure 3B). The distribution and frequency of insulin granules in LP MIN6 cells were typical. However, there were reduced insulin granules per field of view in HP cells (Figure 3C, representative insulin granules are indicated with an arrow).

To determine whether HP MIN6 cells might be consuming ATP due to increased cell proliferation, flow cytometry analysis of BrdU incorporation and 7AAD staining in was performed. HP cells had a lower percentage of cells in S phase of DNA replication (5.4% versus 11.9%, $p<0.001$, Figure 3D). This suggests that HP cells were not utilizing ATP by more rapid cell cycling.

Glucose Oxidation, Glucose Uptake, and Lipid Oxidation

Glucose uptake was measured using the glucose analogue 2-deoxy-[1,2-^{3}H]-glucose. In cells where there is active glucose uptake, such as differentiated C2C12 myotubes, there is ~50% decrease in 2-deoxy-[1,2-^{3}H]-glucose uptake from basal to high glucose conditions, due to dilution by 'cold' non-radioactive glucose [20]. Figure 4A shows an estimation of total glucose uptake in the cells (i.e. including calculated uptake of non-radioactive glucose uptake plus 2-deoxy-[1,2-^{3}H]-glucose). Interestingly, HP cells had approximately 3-fold higher 2-deoxy-[1,2-^{3}H]-glucose uptake at basal glucose versus LP cells ($p<0.01$) but decreased uptake at 25 mM glucose suggesting that their uptake was at a greater proportion of maximal capacity at baseline

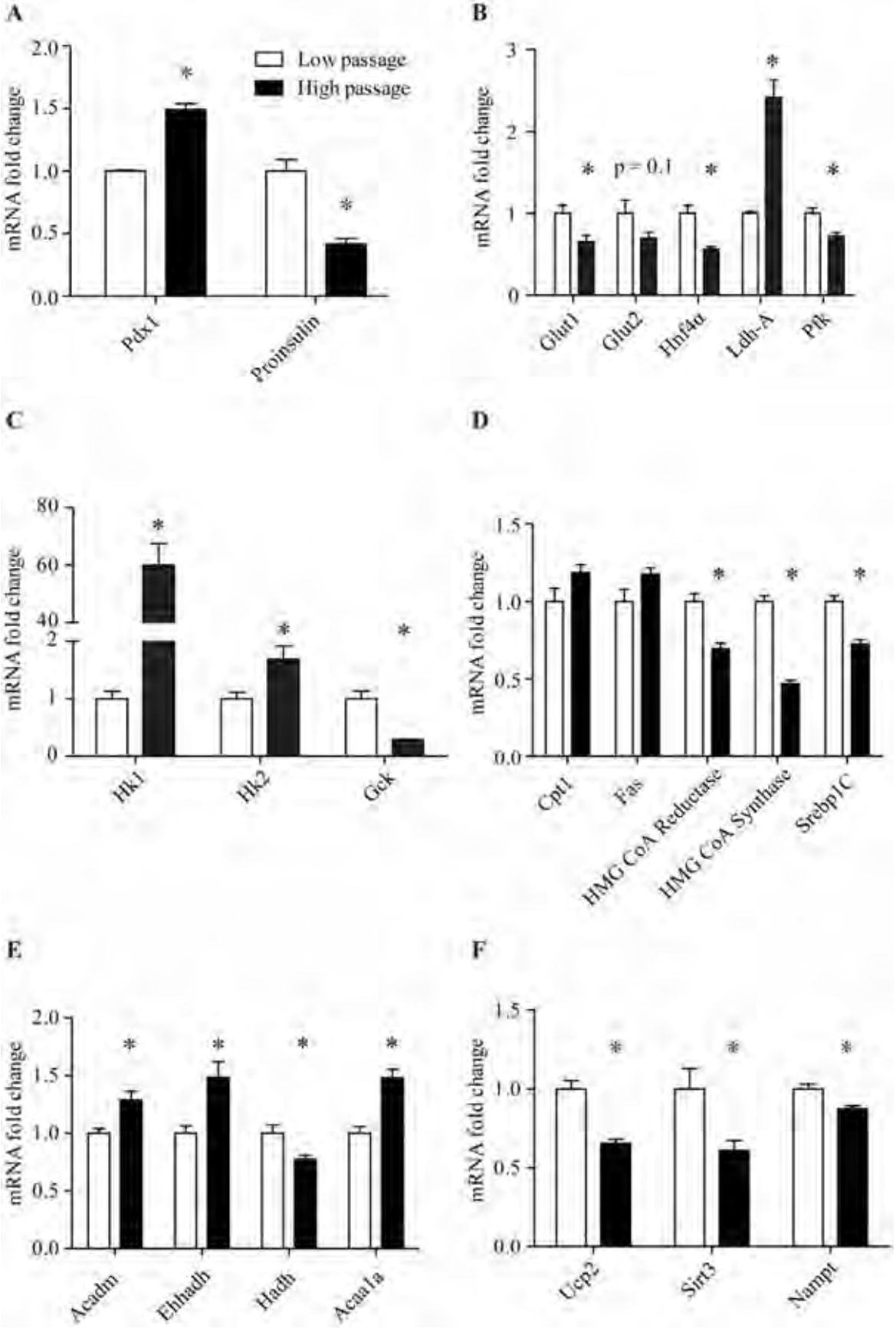
A
mRNA fold change
Low passage
High passage
Pdx1
Proinsulin
B
mRNA fold change
p = 0.1
Glut1
Glut2
Hnf4α
Ldh-A
Pfk
C
mRNA fold change
Hk1
Hk2
Gck
D
mRNA fold change
Cpt1
Fas
HMG CoA Reductase
HMG CoA Synthase
SrebpIC
E
mRNA fold change
Acadm
Ehhadh
Hadh
Acaa1a
F
mRNA fold change
Ucp2
Sirt3
Nampt

Figure 5. Gene expression in HP and LP MIN6 cells. A) HP MIN6 cells had increased expression of *Pdx1* and reduced expression of *proinsulin*. B) HP MIN6 cells had reduced expressions of the glucose transporter *Glut1* and the key glycolytic gene *Pfk*. There was a greater than 2-fold increase in the glycolytic gene *Ldh-A* in high passage MIN6 cells. C) HP MIN6 cells had increased expressions of *Hk1* and *Hk2*, accompanied with a decrease in *Gck*. D) HP MIN6 cells also had reduced expressions of cholesterol synthesizing genes *HMG CoA reductase* and *HMG CoA synthase*, and the transcription factor for lipid synthesis *Srebp1C*. E) HP MIN6 cells had increased expression of lipid oxidation genes *Acadm, Ehhadh, Acaa1a* and increased *Hadh* expression. F) HP MIN6 cells had reduced *Ucp2*, *Sirt3*, and *Nampt* expression. Error bars are ± SEM and n = 3.
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(Figure 4A). Both low and HP cells had a significant increase in calculated total glucose (radioactive plus non radioactive) uptake from 1 to 25 mM glucose which was ~50% greater in LP cells, although not significant (Figure 4A). However, Figure 4B shows the fold-change in total glucose uptake compared to their respective basal levels. LP cells had ~38-fold increase from 1 to 25 mM glucose whereas HP cells only had ~7-fold change ($p<0.05$).

Glucose oxidation was measured by the amount of $^{14}CO_2$ production from the breakdown of D-[U-^{14}C]-glucose. LP cells exhibited a dose dependent increase in glucose oxidation with ~28-fold increase from 1 to 25 mM glucose ($p<0.001$). Consistent with their increased basal glucose uptake, HP cells had an 8-fold higher basal glucose oxidation ($p<0.001$). However, they only displayed a 1.8-fold increase in glucose oxidation at 25 mM glucose. This resulted in 50% lower absolute glucose oxidation in high versus LP MIN6 cells (Figure 4C). Figure 4D shows the fold changes in glucose oxidation at 1 and 25 mM glucose.

Lipid oxidation provides an important source of energy to β-cells. It was measured by $^{14}CO_2$ produced from the breakdown of [1-^{14}C]-palmitic acid. In LP cells, lipid oxidation was high at 1 mM glucose and as expected [21], was markedly reduced at 25 mM glucose by ~6-fold ($p<0.0001$, Figure 4E). In contrast, basal lipid oxidation in HP MIN6 cells was low ($p<0.0001$) and there was no significant change at 25 mM glucose (Figure 4E).

To determine whether the loss of basal lipid oxidation was important for the phenotype of HP MIN6 cells, bromopalmitate was used to inhibit lipid oxidation. Higher concentrations of bromopalmitate caused rapid cell death, indicating the importance of lipid oxidation (data not shown). There was an indication of increased insulin secretion with the addition of 0.0625 μM bromopalmitate for 2 hours (Figure 4G), however, we think this is still due to toxicity as the raw data without correction for total insulin content negates the increase (Figure 4H).

Lactate

To determine if the alterations in glucose metabolism in HP MIN6 cells also involved in increased shunting of glucose into lactate synthesis rather than oxidative metabolism, we measured expression of *Ldh-A* and lactate. No significant differences in lactate were observed between 1 and 25 mM glucose in LP MIN6 cells (Figure 4F). HP cells had a non-significant decrease at 1 mM glucose and a significant increase at 25 mM glucose (Figure 4F). This was still lower than the LP cells. HP MIN6 cells had a 2-fold increase in *Ldh-A* expression ($p<0.00001$, Figure 5A).

Gene Expression between Low and High Passage MIN6 Cells

The mRNA expression of the pancreatic transcription factor *pancreatic duodenal homeobox gene-1* (*Pdx1*) and *proinsulin* were examined. HP MIN6 cells had ~50% increase in *Pdx1* expression ($p<0.00001$) and ~60% decrease in *proinsulin* expression ($p<0.00001$, Figure 5A). HP MIN6 cells had reduced expression of mRNAs encoding the glucose transporter *Glut1*, *Hnf4α*, and *phosphofructokinase* (*Pfk*, $p<0.05$, Figure 5B). HP MIN6 cells had increased expressions of *hexokinase (Hk) 1* and *2*, with *Hk1* increased approximately 60-fold ($p<0.01$, Figure 5C). The key glycolytic gene *glucokinase* (*Gck*, also known as hexokinase IV) was decreased in HP cells ($p<0.01$, Figure 5C).

Analysis of lipid handling genes revealed that HP cells had reduced expression of the important lipid synthesis transcription factor *Srebp1c* ($p<0.0001$, Figure 5D) and decreased cholesterol synthesizing genes *HMG CoA reductase* and *HMG CoA synthase*. HP cells had lower expression of *L-β-hydroxyacyl coA dehydrogenase (Hadh)* and higher *acyl-coA dehydrogenase medium chain (Acadm)*, *enoyl coA hydrotase (Ehhadh)*, and *acetyl-coA acyltransferase 1a (Acaa1a)* (Figure 5E).

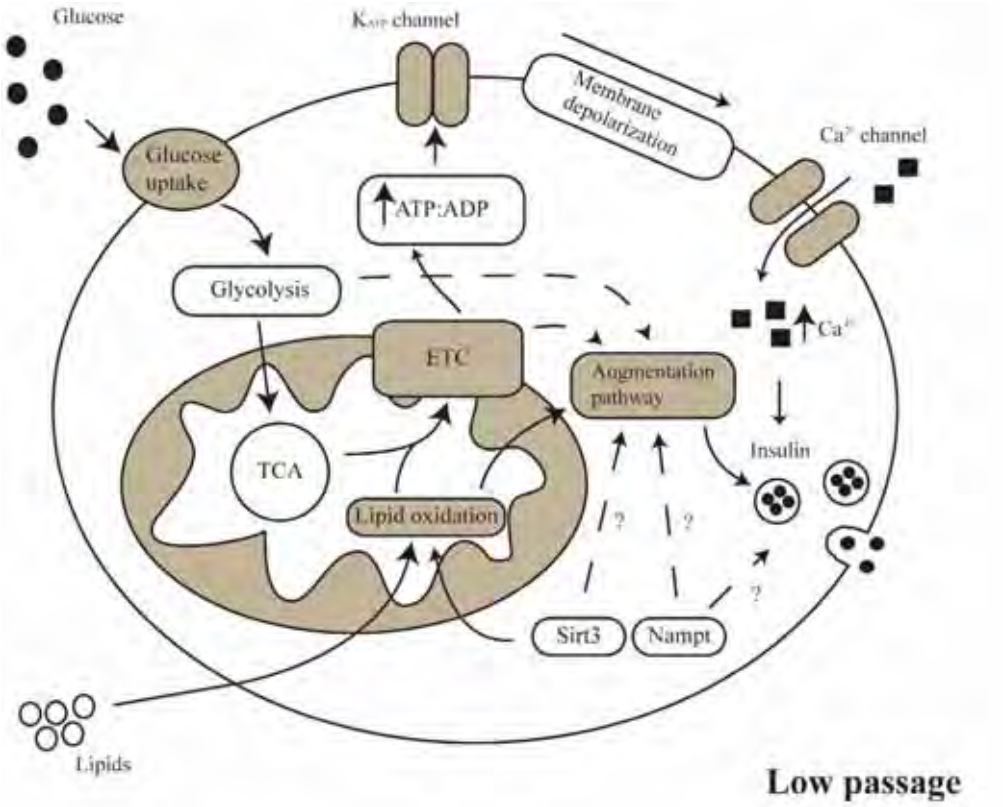
The mitochondrial uncoupling gene, *Ucp2*, was decreased by 35% in HP MIN6 cells ($p<0.0001$, Figure 5F). Only very low levels of *Ucp1* mRNA were present in both LP and HP cells and did not differ (data not shown). *Nampt* has been reported in other tissues to regulate *Sirt3* [22] and *Sirt3* has been shown to regulate lipid oxidation [23]. Expression of both *Nampt* and *Sirt3* was significantly decreased in HP MIN6 cells (Figure 5F).

Discussion

Subjects with T2D lose first phase insulin secretion early in the natural history of disease progression [9,24–26]. In this study, we used MIN6 cells to examine defects in first phase insulin secretion. With high passage these cells had complete loss of first phase insulin secretion and an overall impairment in GSIS. Previous studies in LP and HP MIN6 cells have shown both expression and protein level changes [12–14]; however this is the first report to profile metabolic changes in these cells. HIT-T15 cells, another clonal beta-cell type, are also known to lose both first and second phase GSIS with increased passage. This was attributed to decreased insulin gene expression and insulin content and was suggested to be related to constant exposure to 11 mM glucose [27]. Our HP MIN6 cells displayed similar characteristics, with reduced insulin granule formation and *proinsulin* mRNA expression.

HP cells responded normally to KCl, which works by closing the K_{ATP} channels, downstream of glucose metabolism. Normal KCl-stimulated insulin secretion clearly indicated that the defects in first phase insulin secretion are not in insulin synthesis or secretion capability but lie upstream. L-arginine stimulation of insulin secretion was retained in HP MIN6 cells, but to a lesser extent than LP cells. L-arginine stimulates insulin by inducing Ca^{2+} release from mitochondria via the actions of nitrogen oxides in the presence of glucose [28,29]. These changes again suggest that the defects lie upstream of the K_{ATP} channels.

The lack of an increase in early insulin secretion in HP MIN6 cells was consistent with the failure of increase in intracellular ATP concentration with glucose stimulation. Increased ATP:ADP ratio is crucial in beta-cells as this precedes a cascade of steps necessary for insulin secretion [2–4]. HP MIN6 cells had significantly decreased intracellular ATP at 5 minutes after glucose stimulation but the mechanism or the significance of this is unknown. It is possible that ATP consuming events such as glycolysis could be increased shortly after glucose stimulation without subsequent ATP generation but this needs to be verified. However, impaired high-glucose stimulated ATP was ultimately a result of decreased


Glucose
K_{ATP} channel
Membrane depolarization
Ca^{2+} channel
Glucose uptake
ATP:ADP
Glycolysis
ETC
Ca^{2+}
Augmentation pathway
TCA
Insulin
Lipid oxidation
?
?
?
Sirt3
Nampt
Lipids
Low passage

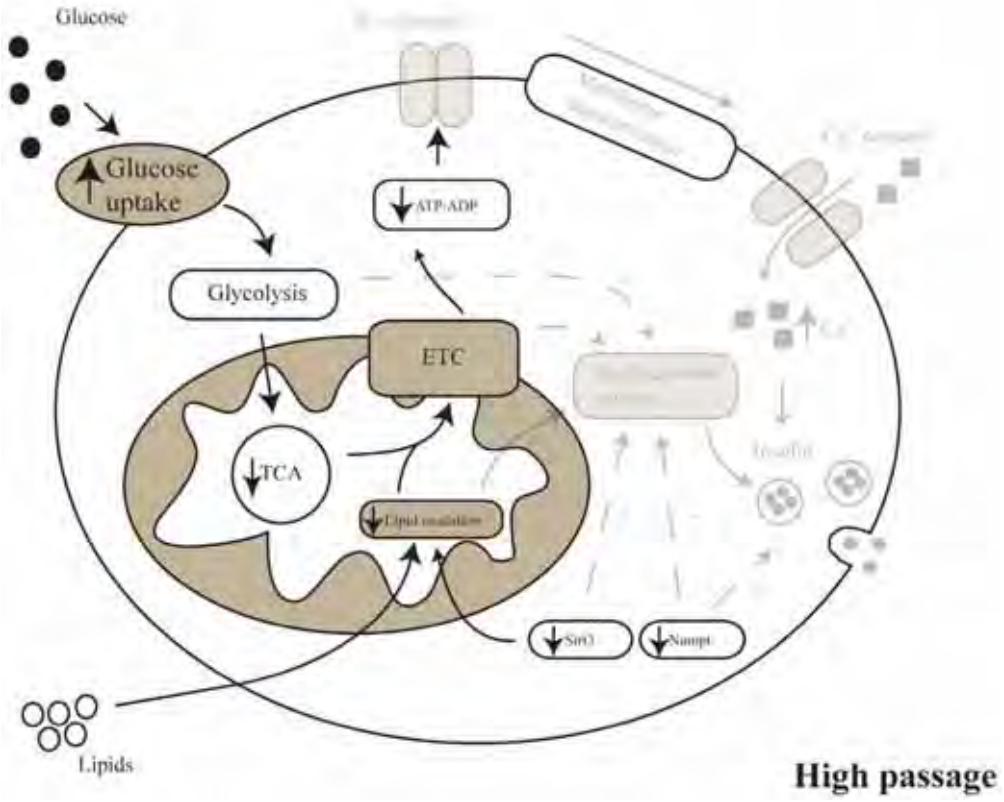

Glucose
Glucose uptake
ATP:ADP
Glycolysis
ETC
TCA
Lipids
High passage

Figure 6. Model of insulin secretion in LP and HP MIN6 cells. HP MIN6 cells increased glucose uptake at 1 mM glucose and decreased expression of some glycolytic genes. Glucose oxidation is decreased compared to LP MIN6 cells when stimulated with glucose, leading to reduced intracellular ATP content and impaired insulin secretion. Lipid oxidation was also reduced. Reduced *Sirt3* and *Nampt* gene expression may be responsible for the impaired metabolism of glucose, ultimately leading to reduced insulin secretion.
doi:10.1371/journal.pone.0040868.g006

glucose uptake and impaired glucose oxidation. This was associated with a significant decrease in *Glut1* and a trend to decreased *Glut2*. Glut2 is the main glucose sensor in the rodent beta-cell due to its high K_m [30]. There was also a reduction in the rate limiting glycolytic enzymes *Gck* and *Pfk*, both of which are important in the provision of substrates to the TCA cycle and oxidative phosphorylation.

Mitochondrial oxidative phosphorylation plays an important role for insulin secretion, as it provides much of the needed ATP to change the ATP:ADP ratio [31–33]. The question of the relative contribution of mitochondrial oxidative phosphorylation versus other pathways in physiological insulin secretion remains controversial [34–37]. Regardless, in order for oxidative phosphorylation to be able to generate ATP, substrates must be provided to it by the glycolytic pathway and the TCA cycle. This flow of substrates could be inhibited by high *Ldh-A* expression. Ldh-A converts pyruvate to lactate and NAD^+ and thus decreases pyruvate. Overexpression of *Ldh-A* in MIN6 cells has been shown to attenuate GSIS [38]. However, lactate in HP MIN6 cells was actually decreased, suggesting that this was not a mechanistic change. There was no change in oxygen respiration rates as measured by a Clark-type oxygen electrode (data not shown).

Some lipid synthesis genes were also down-regulated in HP MIN6 cells, including the important transcription factor *Srebp1c*. Lipids, as well as glucose, are a major source of ATP. Per Mole of substrate, lipid yields more ATP than glucose with up to 136 molecules of ATP generated per palmitate molecule versus 30–36 ATP per glucose molecule. Rough calculations estimating the amount of ATP able to be produced from glucose and lipid oxidation indicate that HP MIN6 cells produce more intracellular ATP compared to LP MIN6 cells at 1 mM glucose. This suggests that HP MIN6 cells are either utilizing much more ATP at 1mM glucose or they are wasting energy. HP MIN6 cells, morphologically different to LP MIN6 cells, were not utilizing extra energy in increased cell proliferation as they had a slower rate of cell division, as evidenced by FACS analysis of the BrdU uptake.

The uncoupling proteins function by dissipating the energy from glucose/lipid oxidation as heat rather than flowing through the electron transport chain to produce ATP [39]. The uncoupling protein Ucp1 is the classic and most well known UCP out of five (Ucp1– Ucp5) but is predominantly expressed in brown adipose tissue [40,41]. There were very low levels of *Ucp1* expression in both low and HP MIN6 cells and these were not significantly different. We measured *Ucp2* expression as this has been proposed to be major factor in obesity, beta cell dysfunction, and type 2 diabetes, negatively regulating insulin secretion [42]. Recent findings have proposed that Ucp2 does not act as an uncoupler and does not contribute to adaptive thermogenesis [41,43–45]. In fact, a previous study has shown that a decrease in intracellular ATP content down-regulated *Ucp2* expression in mouse hepatocytes [46] and mice with homozygous knockout of *Ucp2* have impaired beta cell function, possibly due to increased oxidative stress [47]. Interestingly, HP MIN6 cells had a 35% lower expression in *Ucp2*. The decreased *Ucp2* in HP MIN6 may be secondary to the markedly decreased ATP content.

Recent findings have indicated the enzyme nicotinamide phospho-ribosyl-transferase (Nampt) and nicotinamide adenine dinucleotide (NAD) biosynthesis in insulin secretion and metabolism [48,49]. The enzyme Nampt is the rate limiting step in NAD biosynthesis and mice with a heterozygous deletion of *Nampt* have been shown to have impaired glucose tolerance and isolated islets have reduced GSIS [49]. There is also reduced expression of Sirt3 in streptozotocin induced diabetic mice [50]. The sirtuins are a family of deactetylases and mono-ADP-ribosyltransferases, of which there are seven in mammals, that use NAD as a substrate [51]. It has been previously reported that Sirt1 regulates insulin secretion by repressing *Ucp2* in β-cells [52] but there are no reports regarding Sirt3 and beta cell function. HP MIN6 cells had significantly reduced expression of both *Nampt* and *Sirt3*.

Sirt3 regulates mitochondrial fatty acid oxidation in other tissues and $Sirt3^{-/-}$ mice have reduced ATP levels in various tissues [23,53]. HP MIN6 cells had significantly reduced *Sirt3* expression and reduced lipid oxidation, suggesting that decreased Sirt3 may be driving this.

As lipid oxidation at 1mM glucose failed to provide the cell with ATP, we believe that HP MIN6 cells attempted to compensate for this by increasing glucose uptake and oxidation in the basal state. To determine the importance of basal lipid oxidation, it was blocked with the non-metabolizable lipid bromopalmitate, but this caused cell death and further reductions in insulin secretion.

This data shows that HP MIN6 cells have a very different metabolic profile compared to LP cells. A higher proportion of the HP MIN6 cells had aneuploidy (data not shown) which in other cell lines is correlated with genetic instability [54,55]. Clearly, some of these metabolic changes are causative and some are compensatory in response to a failure in increasing intracellular ATP. It is interesting to note that many of these changes also occur in patients with T2D - impaired insulin response to L-arginine [56], decreased islet glucose oxidation [57], increased whole body glucose uptake at basal glucose levels [58], decreased *Gck*, *Hnf4α*, and *Pfk*, and increased *Pdx1* expression [19,57].

We hypothesize that reduced *Sirt3* and *Nampt* expression are a potential mechanism underlying these metabolic changes, contributing to the decreased lipid oxidation and decreased *Glut1* and some glycolytic gene expression. This led to impaired glucose uptake and a decrease in glucose oxidation (Figure 6).

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Author Contributions

Conceived and designed the experiments: KC CJN NT JEG. Performed the experiments: KC VD NT NH. Analyzed the data: KC VD CJN NT SA JEG. Contributed reagents/materials/analysis tools: CJN VD NT JEG. Wrote the paper: KC JEG. Support and review of manuscript: SA.

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First Phase Insulin Secretion and Type 2 Diabetes

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Abstract: Type 2 diabetes (T2D) is a metabolic disorder characterised by the inability of β-cells to secrete enough insulin to maintain glucose homeostasis. Pancreatic β-cells secrete insulin in a biphasic manner, first and second phase insulin secretion, and loss of first phase insulin secretion is an independent predictor of T2D onset. Restoration of first phase insulin secretion has been shown to improve blood glucose in T2D by suppressing hepatic glucose production and priming insulin sensitive tissue to more readily take up glucose and has thus prompted numerous studies into its regulation. First phase insulin secretion is initiated primarily by the classical triggering pathway, a complex system comprised of multiple stimulatory signals. Recent studies have identified a number of novel regulatory factors that are crucial for first phase insulin secretion and glucose homeostasis. These include, among others, hypoxia inducible factor 1α, von Hippel-Lindau, factor inhibiting HIF, nicotinamide phospho-ribosyl-transferase, and the sirtuin family. This review will outline how first phase insulin secretion is initiated and detail some of the recent findings in its regulation.

Keywords: Aryl hydrocarbon nuclear translocator, glucose homeostasis, hypoxia inducible factor-1α, insulin secretion, regulatory genes, type 2 diabetes.

INTRODUCTION

Type 2 diabetes (T2D) is characterised by the inability of β-cells to secrete enough insulin to maintain glucose homeostasis, usually accompanied by insulin resistance: impaired action of insulin on target tissues. T2D is a multi-factorial disease, the onset of which is likely to be environmental in a permissive genetic background. Thus, environmental influences such as increased caloric consumption and reduced physical activity are clearly involved [1, 2], and recent evidence from genome wide association studies (GWAS) have identified a number of susceptibility loci and polymorphisms which together increase the risk of developing T2D [3, 4].

Pancreatic β-cells secrete insulin in a biphasic manner, defined as first and second phase (Fig. **1**). It is thought that the initial insulin spike is crucial for glucose homeostasis. Studies have shown that loss of first phase insulin secretion is an independent predictor of T2D [5-9]. Disease states associated with impairment in first phase insulin secretion such as Huntington's and Alzheimer's diseases are also associated with increased risk of diabetes [10, 11]. Before the development of frank diabetes, people with fasting hyperglycaemia lack first phase insulin secretion [12] and people with impaired glucose tolerance (IGT) have reduced plasma insulin levels after a glucose load [13-15]. This review will focus on first phase insulin secretion in T2D and recent advances on how it is regulated.

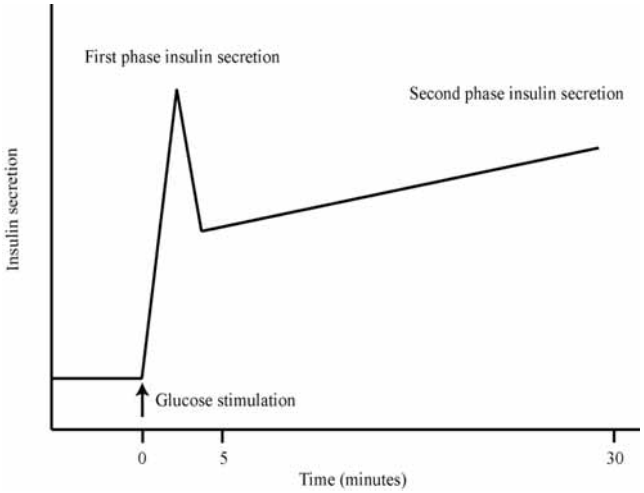

Fig. (1). First and second phase insulin secretion in the β-cell. First phase insulin secretion occurs very rapidly, usually within 10 minutes of glucose stimulation. Second phase insulin secretion is more gradual and long lasting, occurring 25 – 30 minutes after glucose stimulation.

First phase insulin secretion occurs very rapidly, with peak values achieved 1-2 minutes after glucose stimulation [16]. Quite differently, second phase insulin secretion is more gradual and long lasting, usually

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reaching a plateau 25-30 minutes after stimulation in people with normal glucose tolerance [16] (Fig. **1**). While both phases of insulin secretion play an important role in glucose homeostasis, the relative importance of first phase insulin secretion may be greater. Studies in humans where first phase but not second phase insulin secretion have been artificially blocked by an infusion of somatostatin had significantly worse glucose tolerance compared with control conditions [17].

In addition to the biphasic manner of insulin secretion, effective control of glucose homeostasis requires secretion of insulin to be oscillatory [18]. These oscillations occur every 9 – 14 min in humans *in vivo* [19] while others have reported shorter time periods (5 – 8 min) in isolated rat islets [20]. The oscillatory nature of insulin secretion is due to oscillations in glycolysis and its regulatory enzymes [21, 22]. Subjects with T2D have abnormal oscillations in insulin secretion [23] and together with the fact that islets of T2D patients also have reduced glucose oxidation [24] could contribute to inhibited first phase insulin secretion. Some first degree relatives of people with T2D also have abnormal oscillations [25] and thus would suggest some genetic and / or environmental regulation. Insulin infused with an oscillatory pattern has a greater effect than continuous delivery for individuals with diabetes [18] and as such, the less common pulsatile intravenous insulin therapy (PIVIT) or chronic intermittent intravenous insulin infusion therapy (CIIIT) has been used to greater effect [26, 27]. The exact mechanism(s) as to why it is more effective remains to be elucidated but a proposed theory is that PIVIT allows greater expression of insulin receptors than continuously high insulin levels thus increasing sensitivity in peripheral tissues [27].

MEASUREMENT OF FIRST PHASE INSULIN SECRETION

In order to measure biphasic insulin secretion *in vivo*, the following three methods are commonly employed: a) the intravenous glucose tolerance test (IVGTT), b) the hyperglycaemic clamp [28, 29], and c) mixed meal tolerance tests (MMTT) [30, 31]. The IVGTT is relatively simpler to conduct compared with the hyperglycaemic clamp. It involves a bolus of glucose injected intravenously over a short period (typically 1 minute) and blood samples taken at many, specific time points to measure insulin secretion and/or C-peptide [28]. This method is well suited for testing first phase insulin secretion as after the initial glucose injection, no further glucose is introduced into the system. There are issues with variability in readings of the initial insulin spike as glucose decays differently in each subject depending on their glucose tolerance [32]. In normal individuals, approximately 50 % of secreted insulin is cleared by the liver in the first pass of metabolism. So, if C-peptide is measured, deconvolution analysis can be used to estimate actual insulin secretion.

The hyperglycaemic clamp was first described in 1979 by Ralph DeFronzo and colleagues [33] and is a sophisticated technique which requires time and experienced labour. During this procedure, the subject's glucose level is raised to a pre-determined level, for example 125 mg/dl (7 mM/L) above basal levels by a priming dose of intravenous glucose. The hyperglycaemic plateau is then maintained by a constant infusion of glucose. Insulin concentrations, C-peptide concentrations and the glucose infusion rate (GIR) are used as measures of insulin secretion and action [33]. As the β-cells are being exposed to glucose for a longer period of time, it is a more accurate measurement of both first and second phase insulin secretion and is considered the gold standard for the measurement of insulin secretion *in vivo* [32].

The MMTT, also known as the Boost test, is a relatively simple test and is suitable when performing a hyperglycaemic clamp is not feasible, such as in epidemiologic studies or for following individual changes in insulin secretion [30]. In a MMTT, subjects fast overnight then ingest a liquid meal (e.g. Sustacal^{TM}/Boost^{TM}) with a known proportion of carbohydrates, protein, and fat. Common values are 55 % carbohydrate, 25 % protein, and 20 % fat. Blood samples are taken at specific time points to analyse glucose, insulin, and C-peptide levels [30, 31]. Although not considered the gold standard for the determination of insulin secretion, it represents a more physiological response as the stimulus is delivered orally, and induces the normal incretin response [31]. A recent study showed that MMTT provided comparable results at 15 min after intake to results from a hyperglycaemic clamp in children with normal glucose tolerance [30]. The MMTT is clearly physiologically relevant as the normal means of glucose exposure is oral, not intravenous.

REGULATION OF FIRST PHASE INSULIN SECRETION

The Triggering and Amplifying Pathways

With the importance of first phase insulin secretion in glucose homeostasis, it is necessary to understand its regulation. The classical triggering pathway of insulin secretion in the β-cell has been extensively studied and is reasonably well understood [34-36]. Circulating blood glucose is taken up into β-cells by a glucose transporter called GLUT2 and is metabolised by the glycolytic pathway in the cytosol (Fig. **2**). The glucose sensing enzymatic step is catalysed by the hexokinase glucokinase. Together with the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation process located in the mitochondrion, ATP is formed resulting in an increase in the cellular ATP/ADP ratio and closing of the ATP-sensitive potassium channels (K_{ATP} channels) [34, 36, 37]. The cell membrane then becomes depolarised, leading to the opening of the voltage dependent Ca^{2+} channels (VDCC) [38, 39]. An increase in cytoplasmic Ca^{2+} concentration finally leads to the secretion of insulin [40] (Fig. **1**).

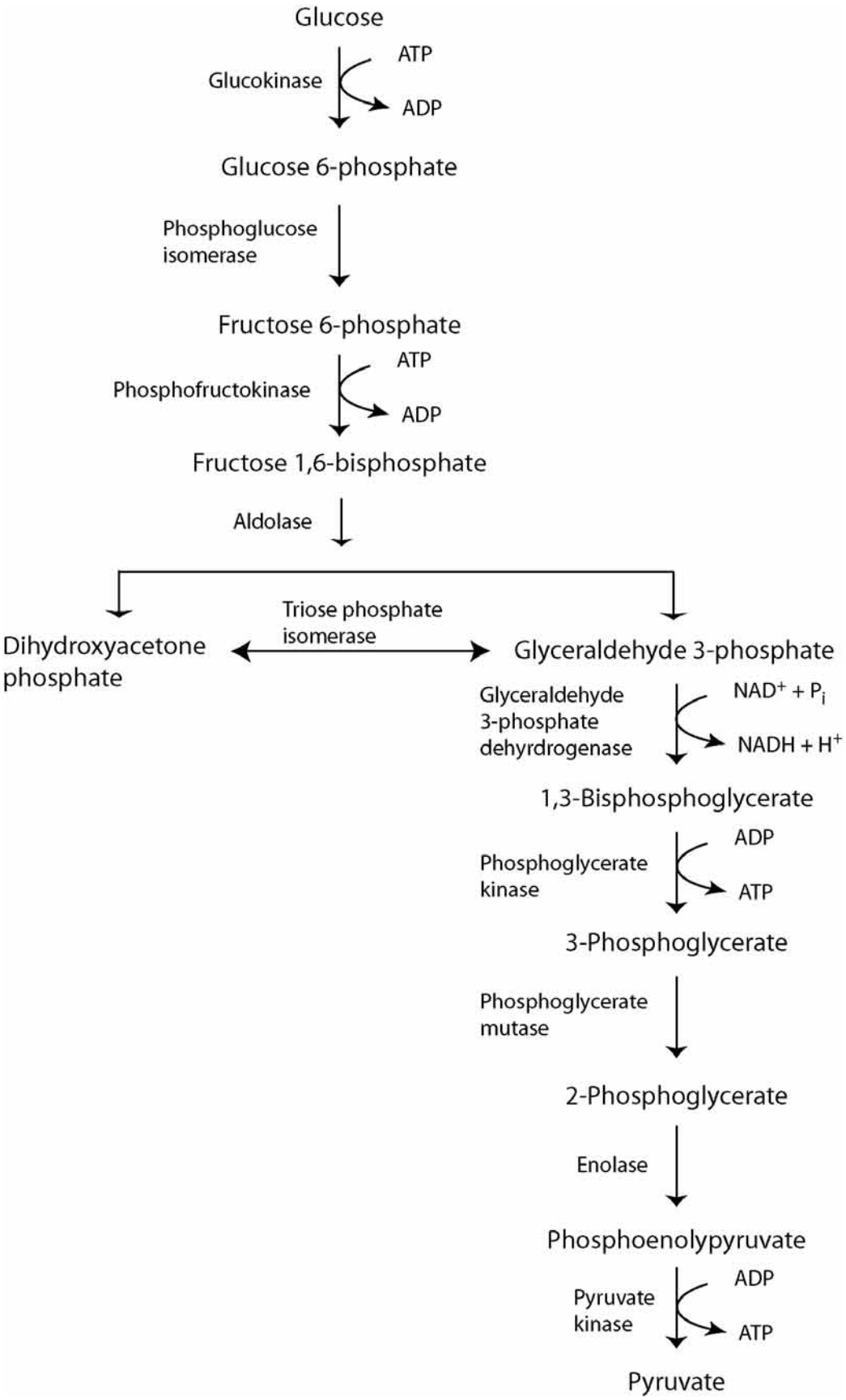

Fig. (2). The glycolysis pathway. The glycolytic pathway consists of ten enzymatic reactions to metabolise glucose to pyruvate. For every one molecule of glucose metabolised, one molecule of NADH and two molecules of ATP are produced.

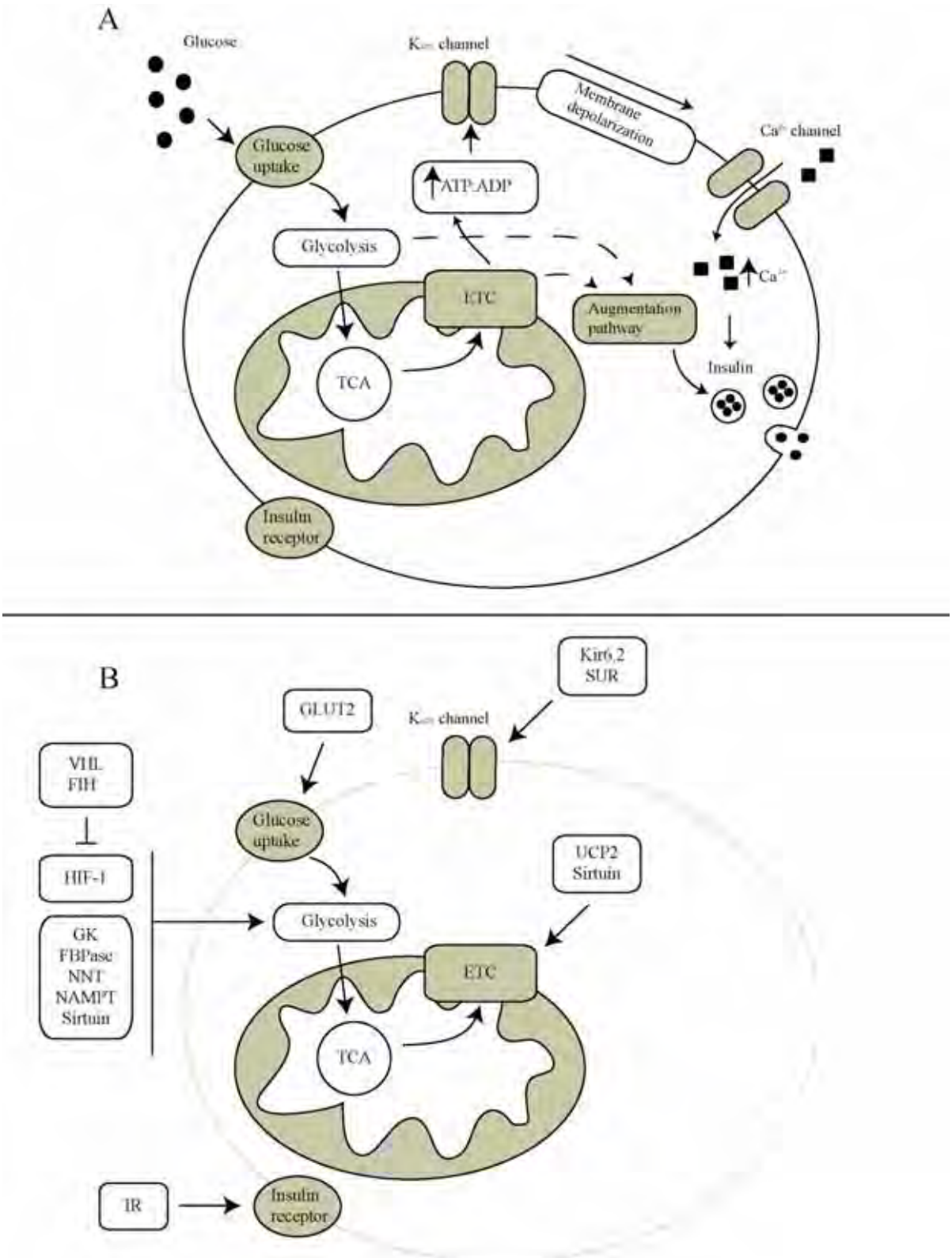

Fig. (3). Model of insulin secretion in the β-cell. (**A**) Glucose is taken up and broken down by glycolysis. This provides substrates for the tricarboxylic acid (TCA) cycle, which in turn provides substrates for the electron transport chain (ETC). ATP generation for the ETC increases the ATP/ADP ratio leading to closure of the K_{ATP} channels. Calcium channels open after membrane depolarisation leading to an increase in intracellular calcium and subsequent insulin exocytosis. The amplification pathway of insulin secretion is less well understood but operates independent of changes in ATP/ADP and the K_{ATP} channels. (**B**) The regulator genes as described in the text and how they interact with the insulin secretory pathway.

The amplifying pathway was identified in 1992 by two groups working independently of each other [41, 42]. These experiments showed that glucose stimulation of insulin secretion was still possible even when K_{ATP} channels were either closed (with KCl) or kept open (with diazoxide), indicating that another pathway must exist. The exact mechanisms of the amplifying pathway are still yet to be fully understood but insulin secretion *via* this pathway seems to involve signalling molecules other than ATP/ADP. Excellent reviews regarding the amplification pathway of insulin secretion have been published [34, 35, 43]. Pyruvate from glucose metabolism can be shuttled off *via* pyruvate carboxylase to form other intermediates which can trigger insulin secretion without the need for closure of K_{ATP} channels. These include NADPH from the malate-pyruvate shuttle and lipid signalling molecules from the malonyl-CoA/LC-CoA pathway [34].

The triggering and amplifying pathways of insulin secretion are not mutually exclusive but complement each other. However, depending on which phase of insulin is occurring, one of the pathways may play a more significant role. It is generally accepted that the first phase insulin secretion is initiated by the triggering pathway and this has much to do with the timing of the event. As stated earlier, first phase insulin secretion occurs within a very short time *in vivo*. It has been suggested that the signalling involved in the amplification pathway may be too slow for this time-frame and thus the triggering pathway is probably the major initiator of first phase insulin secretion [35, 36]. However, a previous study showed that both the triggering and amplifying pathways are involved [44]. This study examined the different signals in the triggering and amplification pathways of insulin secretion and showed that there are shifts in the cytosolic calcium concentration and insulin response curves in first phase insulin secretion, shifts normally associated with the amplification pathway [44].

Insulin Granule Pools

The insulin that is secreted to maintain glucose homeostasis exists as pre-formed insulin granules within the β-cells. Secretory granules reside in two main pools; the docked and reserve pools [45]. The docked pool can be further divided into granules that are immediately releasable and readily releasable, and whether they are primed or un-primed [36]. Estimates suggest approximately 13000 granules in the docked pool and 50 granules per β-cell in the immediately releasable pool in normal mice [36, 46]. The 50 granules docked and immediately releasable are located in close proximity to the calcium channels and are utilised for first phase insulin secretion [46]. The second phase of insulin secretion is thought to be maintained by a subset of approximately 1000 granules which are docked but un-primed [36]. In order for the granules to be primed and readily releasable, they must go through a series of reactions that involve ATP, calcium, and temperature [47, 48].

The Role of Glycolysis and Mitochondrial Oxidative Phosphorylation

Glycolysis occurs in the cell cytosol and is the process by which glucose is converted to pyruvate, yielding a net of two molecules of ATP per molecule of glucose (Fig. **2**). The glycolytic pathway is nearly ubiquitous in cellular organisms and is an important process not only to provide a small amount of ATP but to provide precursor components for the TCA cycle and oxidative phosphorylation [49].

Often referred to as the citric acid cycle or the Kreb's cycle, the TCA cycle produces substrates that will be utilised in oxidative phosphorylation to produce ATP under aerobic conditions [49]. Oxidative phosphorylation occurs within the inner mitochondrial membrane and is the process by which ATP is generated by the transfer of electrons from NADH or $FADH_2$ to oxygen by a series of electron transporters. In essence, this is achieved by the generation of an electron-motive force, conversion into a proton-motive force and using this force to drive ATP synthesising assembly [49].

Mitochondrial oxidative phosphorylation plays an important role for insulin secretion, as it provides much of the needed ATP to change the ATP/ADP ratio [50-52]. It has been clearly established that mitochondrial dysfunctions can lead to diabetes [53-55]. Recent experiments have shown that mice deficient in the pancreatic duodenal homeobox 1 (*PDX1*), a β-cell master gene, displayed defective insulin secretion due in part to suppression of the mitochondrial transcription factor A (*TFAM*) [53]. *TFAM* is crucial for the stability and transcriptional activity of mitochondrial DNA [56].

However, the question of the relative contribution of mitochondrial oxidative phosphorylation versus other pathways in physiological insulin secretion remains controversial [57-61]. One such argument is in regards to reactive oxygen intermediates (ROI), which include hydroxyl radicals, hydrogen peroxide, superoxide, and singlet oxygen produced in the mitochondria [62], peroxisomes [63], and plasma membrane-associated NAD(P)H oxidases [64]. Excess ROI levels lead to suppression of insulin secretion [65, 66], however, absent or very low ROIs also inhibit insulin secretion. Low but not excessive ROIs stimulate insulin secretion [65, 67].

While the fact the mitochondrial derived ROI's having a role in insulin secretion is not disputed, some researchers propose that other sources of ROI's may contribute to a larger extent towards insulin secretion than previously thought. Rotenone, an inhibitor of mitochondrial complex I, stimulates insulin secretion at basal glucose levels [68] and taken together with a previous study showing that rotenone inhibition of mitochondrial activity reduces mitochondrial ROI production in cardiac cells [69] may indicate that extra mitochondrial sources of ROI are responsible for the increased insulin secretion.

Regulatory Genes

Identifying genes that play a role in insulin secretion is an important research area which has the potential to lead to targets for the treatment of diabetes. A summary of some of these regulatory genes is outlined below.

Genome Wide Association Studies

There is substantial evidence from studies both in families [70] and monozygotic twins [71] for a strong genetic component of type 2 diabetes. Correspondingly, recent studies using large scale, genome wide association studies (GWAS) have identified ~40 susceptibility loci. Polymorphisms within these *loci* increase the risk of type 2 diabetes and, in the majority of cases; this is through changes in beta-cell function [3, 4, 72, 73]. The identification of such susceptibility *loci* has prompted a number of subsequent studies which have sought to explore the molecular mechanisms through which the implicated genes may impact on β-cell function or survival using rodent models and cellular systems [74-77]. It is unclear which of these genes have an impact on first phase insulin secretion yet but polymorphisms in the transcription factor 7-like 2 (*TCF7L2*) are associated with reduced first phase insulin secretion [78].

Glucose Transporter 2

To perform its biological function, glucose needs to be transported across the plasma membrane and this is facilitated by a family of glucose transporters; GLUT1-12. The members of the GLUT family are highly related but they differ in their tissue specificity and function [79]. GLUT2 is highly expressed in the liver and pancreatic β-cells and is an important part of the glucose sensing mechanism necessary for insulin secretion due to its low affinity and high K_m for glucose [80]. Reduced expression of *GLUT2* has been found in animal models of type 2 diabetes including the neonatal streptozotocin rat [81], the diabetic Zucker rat [82], and the db/db mouse [83]. Mice that have a homozygous deletion of *GLUT2* display characteristics of type 2 diabetes, including a loss of first phase but preserved second phase insulin secretion [84]. The relative importance of GLUT2 in humans is less clear. People with some mutations in *GLUT2* develop Fanconi-Bickel syndrome, which is associated with glucose intolerance and diabetes [85]. A V197I mutation in human *GLUT2* has been shown to abolish glucose transport [86] and thought to be associated with T2D [87]. However, in a small sample of human control and diabetic subjects, no change in *GLUT2* mRNA or protein expression was observed [88, 89] and a recent study has suggested that GLUT1 and GLUT3 are the main glucose transporters in human pancreatic β-cells [90]. Indeed, *GLUT1* expression in human islets was higher than *GLUT2* [89]. Therefore, despite the very clear effects of *GLUT2* disruption in mice with regards to insulin secretion, human GLUT2 may not have the same roles as it does in rodents.

Glucokinase

β-cells secrete insulin in response to increases in blood glucose levels and thus they need to be able to sense changes in glucose concentrations. Glucokinase (GK) is the initial rate determining step of the glycolytic pathway (Fig. **2**), phosphorylating glucose to glucose 6-phosphate. Glucokinase is thought to be the main glucose sensor for β-cells due to its high K_m of approximately 10 mM and high specificity for glucose [91]. This is supported by the fact that heterozygous mutations in *GK* cause maturity onset diabetes of the young (MODY). In humans, *GK* mutations account for 50 % of cases of MODY [92-94] and cause a right-shift in the GSIS curve.

A number of studies have been performed to identify the role of GK in β-cells [91, 95-99]. Mice with homozygous deletions of *GK* are born diabetic and die shortly after birth. However, mice with one functioning allele of GK survive ($GK^{+/-}$) but are hyperglycaemic and display impaired first and second phase glucose stimulated insulin secretion (GSIS) [91, 96]. This was shown to be caused by the inability of glucose to close the K_{ATP} channels and thus inadequate depolarisation of the β-cell [98]. Mice with targeted deletion of *GK* only in the β-cells display a very similar insulin secretory profile to that of the $GK^{+/-}$ mice [95]. Interestingly, as *GK* is only expressed in the liver and pancreatic β-cells, rescue of $GK^{-/-}$ mice was possible by overexpressing *GK* in the β-cells only, suggesting that β-cell GK was more important than liver GK for glucose homeostasis and survival [91].

K_{ATP} Channels

An important step in the triggering pathway of insulin secretion is the closure of the K_{ATP} channels, allowing for subsequent membrane depolarisation and insulin exocytosis. The K_{ATP} channels in general are comprised of a K^+ inward rectifier (Kir6.1 or Kir6.2) and a sulfonylurea receptor (SUR1 or SUR2), with SUR1 and Kir6.2 making up the pancreatic β-cell K_{ATP} channels [100]. It has been shown that disruption of either of these subunits leads to impaired insulin secretion in mice [101, 102] but conversely leads to hypoglycaemia and increased insulin secretion in people [103-105].

Both $SUR1^{-/-}$ and $Kir6.2^{-/-}$ mice have been generated and display similar phenotypic changes [101, 102, 106, 107]. Glucose stimulated insulin secretion, both first and second phase, was impaired in the $SUR1^{-/-}$ and $Kir6.2^{-/-}$ mice. Interestingly, euglycaemia was maintained by these animals, as the $Kir6.2^{-/-}$ mice had no deviations in their glucose tolerance compared to control littermates [102], and the $SUR1^{-/-}$ had normal random fed glucose levels (albeit with slightly impaired glucose tolerance) [101]. This was achieved through different mechanisms. The $Kir6.2^{-/-}$ mice was able to maintain normal glucose tolerance in the face of reduced first and second phase GSIS due to an increased insulin sensitivity in peripheral tissue [102]. However, the $SUR1^{-/-}$ mice did not display any changes to insulin sensitivity and the

authors postulated that euglycaemia was maintained in a fed state by insulin release *via* K_{ATP} channel independent pathways [101].

Fructose-1,6-Bisphosphatatase

Fructose-1,6-bisphosphatase (FBPase) is a gluconeogenic enzyme that catalyses the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate. The expression of *FBPase* has been shown to be increased in a number of diabetic mouse models including the New Zealand Obese (NZO) and the BTBR mice [108, 109] and this increase in expression may be induced by an increase in fatty acids [110, 111]. Insulin secretion was reduced in mice and the MIN6 cell model with an overexpression of *FBPase* and it is physiologically relevant to human T2D as human T2D islets have an increase in *FBPase* expression [111]. *FBPase* overexpressing MIN6 cells had reduced glucose utilisation and metabolism, resulting in a reduction in ATP generation, all of which contribute to the observed reductions in insulin secretion [111].

Hypoxia Inducible Factor-1

We have identified a pair of transcription factors; hypoxia inducible factor-1α (HIF-1α) and the aryl hydrocarbon receptor nuclear translocator (ARNT), which play a role in first phase insulin secretion. ARNT and HIF-1α forms the HIF-1 transcription factor, first identified by Semenza and Wang in 1992 [112]. It was first brought to attention as a regulator of insulin secretion by Gunton and colleagues [89] when it was described that *ARNT* was markedly reduced in islets of T2D patients. Mice with β-cell specific knockout of *ARNT* had reduced glucose tolerance and absent first and impaired second phase GSIS both *in vivo* and in isolated islets. This was supported by evidence in siRNA knock down of *ARNT* in the MIN6 cell culture line, which showed similar results. The reduced insulin secretion was accompanied with decreased expression of *glucose-6-phosphoisomerase (G6PI)* and *aldolase (ALDO)*, two key glycolytic genes [89]. *ARNT* was previously thought to be ubiquitously and constitutively expressed, but it has since been shown that the carbohydrate-responsive element-binding protein (ChREBP) is a negative regulator [113].

Subsequent studies have shown that ARNT's partner HIF-1α also plays an important role in insulin secretion. HIF-1α protein is regulated post translationally in an oxygen dependent manner. Under hypoxic conditions, HIF-1α is stable and can bind to ARNT to commence transcription of target genes. However, at normoxia, HIF-1α is targeted for degradation *via* an oxygen sensitive ubiquitin-proteasome pathway and has a very short half-life [114]. Studies in our lab have shown that mice with β-cell specific knockout of *HIF-1α* and also siRNA knockdown of *HIF-1α* in MIN6 cells displayed many of the same phenotypic changes seen in the *ARNT* knockout/knockdown models described above. Similar to the *ARNT* knockout mice, mice with β-cell specific knockout of *HIF-1α* also has decreased expressions of the key glycolytic genes *G6PI* and *ALDO*, but also of *GK* and *phosphofructokinase* [115]. Both *ARNT* and *HIF-1α* seem to be regulators of glycolysis, and taken together with that fact they have reduced GSIS, would further provide backing for the importance of glycolysis in insulin secretion.

Interestingly, when HIF-1α protein is stabilised with an iron chelator, insulin secretion was improved and *ARNT* gene expression was restored in type 2 diabetic human islets [115]. As such, HIF-1 seems to be an important regulator of insulin secretion and a possible target for therapy.

Von Hippel-Lindau and Factor Inhibiting HIF

Von Hippel-Lindau protein (VHL) is a tumour suppressor and forms a part of the pVHL-elonginB-elonginC (VBC) complex. Under normoxic conditions, the VBC complex binds to HIF-1α protein and targets it for proteosomal degradation [116]. Deletion of *VHL* stabilises HIF-1α protein under normoxic conditions. A number of studies have found that mice with homozygous pancreatic or β-cell specific knockout of *VHL* have impaired insulin secretion and significantly impaired glucose tolerance [117-120]. However, mice with β-cell specific knockout of both *VHL* and *HIF-1α* reversed the *VHL* knockout phenotype and had normal insulin secretion, showing that the massive increase in HIF-1α protein was responsible for the deleterious effects [117].

This raises an interesting scenario in which both deletion of *HIF-1α* and increasing HIF-1α protein (by deleting *VHL*) in mice both impaired insulin secretion. We believe this follows a "Goldilocks" paradigm analogous to the effect of reactive oxygen species (ROS), where too much and too little are both deleterious for β-cell function. Interestingly, HIF-1α is both regulated by and regulates ROS [121-123]. Not enough HIF-1α is detrimental [117-120]. However, increasing HIF-1α protein by using an iron chelator to the point where it is "just right" [115], and not to the levels observed by deleting *VHL*, is beneficial for insulin secretion.

This is supported by a study by Zhang *et al*. investigating the effects of knocking out factor inhibiting HIF-1α (FIH) [124]. Under normoxic conditions, HIF-1α protein is highly regulated at the protein level by hydroxlases, with FIH being one of them. Mice with *FIH* knocked out displayed none of the characteristics of a *VHL* knockout, instead these mice had improved insulin sensitivity and decreased weight, even under a high fat diet challenge [124]. These results are similar to those reported by our group using iron chelation to stabilise HIF-1α protein activity [115]. Additional circumstantial support is provided by the fact that people with VHL syndrome in whom there are heterozygous mutations in VHL appear to be protected from diabetes development, especially after pancreatic resections [125-128]. To our knowledge, glucose tolerance in mice with heterozygous *VHL* defects in the pancreas has not been reported, and would be of particular interest.

Insulin Receptor

The insulin receptor (IR) is a ubiquitously expressed cell surface protein capable of binding insulin with high affinity, setting off a cascade of insulin signalling reactions in peripheral tissues necessary for glucose homeostasis [129]. In humans, heterozygous mutations in *IR* resulting in loss of function any of the heteroterameric receptors including a mutant allele results in leprechaunism, with traits including growth retardation and diabetes [130]. The phenotype is very severe in mice with homozygous *IR* deletion in that they die shortly after birth (48 – 72 hrs) due to diabetic ketoacidosis [131]. Tissue specific *IR* knockout mice have been made. Muscle specific *IR* knockout mice display some of the characteristics of T2D, including increased triglyceride and serum free fatty acids but glucose tolerance remains normal without additional insults [132]. More interestingly, mice with β-cell specific knockout of *IR* display many of the characteristics of T2D, including a loss of first phase but retained second phase insulin secretion [133]. This is of particular importance as it provides a model for T2D development, whereby insulin resistance at the β-cell level (replicated in mice with a β-cell specific knockout of *IR*) leads to reduced first phase insulin secretion, a major characteristic of disease development [133].

Nicotinamide Nucleotide Transhydrogenase

Nicotinamide nucleotide transhydrogenase (NNT) is a nuclear encoded mitochondrial protein responsible for the reduction of $NADP^+$ by NADH and conversion of NADH to NAD^+. This makes it particularly important in terms of insulin secretion because it generates NADPH and thus affecting mitochondrial metabolism [134]. First identified to have a role in insulin secretion by Toye *et al*. [135], *NNT* expression was shown to be significantly lower in C57BL/6J mice, a strain of mice exhibiting impaired glucose homeostasis independent of obesity (including reduced first phase insulin secretion after feeding). In addition, C57BL/6J mice display a 5-exon deletion in NNT, which is not present in other strains of mice including the closely related C57BL/6N [135, 136]. This was subsequently validated by a study showing that mice with mutant forms of *NNT* have reduced glucose tolerance and first phase insulin secretion, with an accompanied increase in glucose utilisation and decreased ATP production [134]. This phenotype was attributed to ROS mediated activation of UCP2 and thus uncoupling of mitochondrial oxidative phosphorylation [134]. In addition to this, upregulation of *NNT* was shown to be a cause for insulin hypersecretion in the DBA/2 mice, a diabetes susceptibility mouse model [137].

Nicotinamide Phospho-Riboysl-Transferase and the Sirtuin Family

Recent findings have implicated the enzyme nicotinamide phospho-ribosyl-transferase (NAMPT) and nicotinamide adenine dinucleotide (NAD) biosynthesis in insulin secretion and metabolism [138, 139]. The enzyme NAMPT is the rate limiting step in NAD biosynthesis. Mice with a heterozygous deletion of *NAMPT* have been shown to have impaired glucose tolerance. Isolated islets from $NAMPT^{+/-}$ mice have reduced glucose stimulated insulin secretion at 15 min after stimulation, a time point representing first phase insulin secretion in *in vitro* studies [139]. The authors found that the deficiency in insulin secretion was due to the defects in nicotinamide mononucleotide (NMN) and nicotinamide adenine dinucleotide (NAD) biosynthesis, possibly altering glycolysis or lipid oxidation as a downstream consequence [139]. The deficiency in NAD would have downstream effects on NAD-dependent enzymes, in particular, the sirtuin family.

The sirtuins are a family of deactetylases and mono-ADP-ribosyltransferases, of which there are seven in mammals, that use NAD as a substrate [140]. It has been previously reported that Sirt1 regulates insulin secretion by repressing *UCP2* in beta cells [141] and over expressing *Sirt1* in β-cells improves first phase insulin secretion [142]. More recently, Sirt4 has also been implicated as having a role in metabolism. Sirt4 is a mitochondrial protein that has been shown to regulate insulin secretion by repressing glutamate dehydrogenase activity, thereby reducing the ability of mitochondria to generate ATP from glutamate and glutamine [143, 144]. The generation of ATP is vital for the triggering pathway of insulin secretion and thus Sirt4 could play an important role in first phase insulin secretion. These are the only members of the sirtuin family which have been directly associated with insulin secretion although emerging evidence may shed some light on the role of Sirt3 in this respect. Reduced expression of Sirt3 in streptozotocin induced diabetic mice has been reported [145].

Uncoupling Proteins

The uncoupling proteins (UCP) function by dissipating the energy from glucose/lipid oxidation as heat rather than flowing through the electron transport chain to produce ATP [146]. The uncoupling protein UCP1 is the classic and most well known UCP out of five (UCP1 – UCP5) but is predominantly expressed in brown adipose tissue [147, 148]. Of the other UCP's, UCP2 has a definite role in insulin secretion, however research has shown conflicting results. On one side of the argument, *UCP2* expression has been proposed to be a major factor in obesity, β-cell dysfunction, and T2D, negatively regulating insulin secretion [149]. In clear contrast to this, a more recent study reported that over-expressing *UCP2* in mice had no effect on insulin secretion [150]. Another study has shown that mice with homozygous knockout of *UCP2* have impaired β-cell function, possibly due to increased oxidative stress [151]. Therefore, even though UCP2 may have a role in insulin secretion, there are both positive and negative impacts in the current literature.

Restoration of First Phase Insulin Secretion

First phase insulin secretion is crucial in maintaining glucose homeostasis and a reduction in this early

phase may be the first detectable signs of β-cell dysfunction and increased risk of T2D. Therefore, restoration of first phase insulin secretion may have a positive impact on disease development. A study in dogs showed that restoration of first phase insulin secretion (even in the absence of second phase) reduced EGP [152]. As the inability to suppress EGP can lead to IGT [152-154], restoration of first phase insulin secretion could be an important first step in the treatment of type 2 diabetes.

Advances in medical techniques have allowed individuals with morbid obesity and T2D to undergo various forms of bariatric surgery to reduce body weight. In many cases, this alleviates or cures T2D [155-158]. These types of surgery may involve a variety of procedures but the general premise is reducing the size of the stomach by using a medical device or removal of a portion of the stomach in morbidly obese patients. While weight loss and the control of diabetes after these types of surgeries has been well established [155-158], a surprising finding is that first phase insulin secretion is also restored, often soon after surgery [155, 158]. The exact mechanisms responsible for this are as yet unknown but it was thought that weight loss alone was not the answer due to the relatively short time frame for improvement in first phase insulin secretion (one month after surgery) [155] and weight loss obtained through dieting was unable to restore first phase insulin secretion in T2D patients [159, 160]. It has been proposed that changes in gastrointestinal hormones, adipokines, gluco- and lipotoxicity could be the mechanisms for the restoration of first phase insulin secretion post surgery [155]. However, a new study shows that calorie restriction itself, and decreased pancreatic fat content may well be the factors associated with improvement [160]. Researchers in this study showed that patients with T2D who went on a calorie restricted diet (total energy intake of 2.5 MJ (600 kcal)/day)) showed improvements in fasting glucose 1 week after the diet and first phase insulin secretion was restored after 8 weeks on the diet [160]. This may have been due to the decrease in total pancreatic fat observed in the dieting patients and despite the small sample size (11 T2D patients and 9 matched control individuals), it provides fascinating insight into the potential mechanisms for the improvements seen in bariatric surgery patients.

SUMMARY

It is clear that first phase insulin secretion has an important role in maintaining glucose homeostasis. The initial insulin response after glucose stimulus primes the system, suppressing HGP in the liver and readies insulin sensitive tissue to take up the incoming glucose. Previous research has shown that loss of first phase insulin secretion is one of the earliest detectable symptoms for T2D onset and emerging research has identified restoration of this phase of insulin secretion may benefit disease sufferers, the most pronounced being post-bariatric surgery. Research has identified many factors that play a role in first phase insulin secretion, including ATP/ADP ratios, ROS, HIF-1, VHL, and members of the sirtuin family (Table **1**). Therapies which increase first phase insulin secretion include weight loss and GLP-1 receptor agonists. More research will uncover even more regulatory genes and therapies and increase our ability to treat type 2 diabetes.

Table 1. A Summary of the Genes Described in the Text and their Effects on Insulin Secretion

Genotype	Effect on Insulin Secretion
β-cell specific knockout of *ARNT*	Decreased [89]
***FBPase* transgenic**	Decreased [111]
***GLUT2*$^{-/-}$**	Decreased [84]
***GK*$^{+/-}$**	Decreased [91]
β-cell specific knockout of *HIF-1α*	Decreased [115]
β-cell specific knockout of *IR*	Decreased [133]
***Kir6.2*$^{-/-}$**	Decreased [102]
***NAMPT*$^{+/-}$**	Decreased [139]
Mutant *NNT*	Decreased [134]
***Sirt1* transgenic**	Increased [141, 142]
***SUR1*$^{-/-}$**	Decreased [101]
Polymorphisms in human *TCF7L2*	Decreased [78]
***UCP2*$^{-/-}$**	Both increased and decreased [149, 150]
β-cell and pancreas specific *VHL-null*	Decreased [117-120]

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

ABBREVIATIONS

ADP = Adenosine diphosphate

ALDO = Aldolase

ARNT = Aryl hydrocarbon receptor nuclear ranslocator

ATP = Adenosine triphosphate

ChREBP = Carbohydrate-responsive element-binding protein

CIIIT = Chronic intermittent intravenous insulin therapy

FBPase = Fructose-1,6-bisphosphatase

FIH = Factor inhibiting HIF

G6PI = Glucose-6-phosphoisomerase

GIR = Glucose infusion rate

GK = Glucokinase

GLP-1 = Glucogon like peptide-1

GLUT = Glucose transporter

GSIS = Glucose stimulated insulin secretion

GWAS = Genome wide association studies

HGP = Hepatic glucose production

HIF-1α = Hypoxia inducible factor-1α

IGT = Impaired glucose tolerance

IR = Insulin receptor

IVGTT = Intravenous glucose tolerance test

K_{ATP} = ATP-sensitive potassium channels

K_m = Michaelis constant

MMTT = Mixed meal tolerance test

NAD = Nicotinamide adenine dinucleotide

NNT = Nicotinamide nucleotide transhydrogenase

NAMPT = Nicotinamide phospho-ribosyl-transferase

PIVIT = Pulsatile intravenous insulin therapy

ROI = Reactive oxygen intermediates

ROS = Reactive oxygen species

siRNA = Short interfering RNA

T2D = Type 2 diabetes

TCF7L2 = Transcription factor 7-like 2

TCA = Tricarboxylic acid

UCP = Uncoupling protein

VBC = pVHL-elonginB-elonginC

VHL = von Hippel-Lindau

VDCC = Voltage dependent calcium channel

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Review

Novel links between HIFs, type 2 diabetes, and metabolic syndrome

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Hypoxia inducible factors (HIFs) are master-regulators of cellular responses to hypoxia, and thus are crucial for survival. HIFs also play a role in regulating cellular processes in β-cells, liver, muscle, and adipose tissue, have effects on the regulation of weight, and play a role in type 2 diabetes (T2D). Indeed, in people with T2D the HIF pathway is dyregulated in major metabolic tissues involved in the pathogenesis of diabetes. This review covers the contrasting, complementary and conflicting effects of decreasing and increasing HIFs in various tissues, and shows that a delicate balance exists between HIF levels and optimal metabolic function. We propose that increasing the activity of HIFs might be a potential therapeutic strategy for treating T2D.

HIFs

Oxygen is essential to the survival of all multicellular organisms. It is a prime substrate in the generation of ATP, the major energy source, but in excess, oxygen leads to the formation of reactive oxygen species (ROS) and oxidative cellular damage. Therefore its homeostasis must be finely tuned. Beyond whole-organism oxygen homeostasis, reviewed in [1,2], recent emerging evidence suggests that dysregulation of the HIF pathway has a role in the pathophysiology of a range of diseases including metabolic syndrome, diabetes, cancer, heart disease and pulmonary vascular disease [3,4]. Several recent papers have reported seemingly conflicting findings in tissue-specific HIF activity, and these make this a complex field. Here we review the regulation of HIFs and their roles in the major metabolic tissues involved in the pathogenesis of diabetes and the metabolic syndrome.

The HIF family and their regulation

The HIF proteins coordinate at the transcriptional level the cellular response to oxygen availability [2]. Since their discovery 15 years ago remarkable progress has been made in understanding the structure and function of these proteins and their role in basic cellular physiology. There are now hundreds of known HIF target genes, including genes encoding proteins involved in angiogenesis, apoptosis, cell cycle progression, glucose uptake, glycolysis, and lipid metabolism [3]. As an indispensable component for normal development, deletion of the family members ARNT (also termed HIF-1β, see Glossary), HIF-1α, or HIF-2α in mice is lethal before birth or perinatally, with the exception of one line of HIF-2α null mice with partial survival on a mixed genetic background [1,5–9].

HIFs are heterodimeric transcription factors that belong to the basic helix-loop-helix Per–ARNT–Sim (bHLH-PAS) family. HIF-1 is composed of two parts, namely ARNT (also named HIF-1β) and HIF-1α, both of which play interdependent roles in transcriptional regulation. HIF-2 is composed of ARNT2 (also termed HIF-2β) and HIF-2α, and similarly HIF-3 is made up of ARNT3 (also known as HIF-3β) and HIF-3α. The HIF α subunits are tightly regulated at the protein level [1,10], as depicted in Figure 1a.

HIFs are rapidly hydroxylated, ubiquitinated and destroyed by proteolysis, giving a half life of minutes. Hydroxylation is carried out by prolyl hydroxylases (PHDs) and an asparagine hydroxylase named FIH1 (factor inhibiting HIF). Once hydroxylated, HIF α subunits associate with the von Hippel–Lindau (VHL) protein, which forms part of an E3 ubiquitin ligase complex. This ubiquitinates HIFs and targets them for proteolysis. The hydroxylases require oxygen, iron and 2-oxoglutarate (also known as α-ketoglutarate) as cofactors. Thus, hypoxia, iron depletion, or decreased 2-oxoglutarate inhibit proteolysis and HIF activity increases. Mutations in the human *VHL* gene cause a syndrome of the same name which is associated with an increased risk of vascular tumors [11]. Mutations affecting PHDs or FIH also increase HIF activity in people.

Glossary

Akt/PKB (protein kinase B): a serine/threonine-specific protein kinase that plays roles in a variety of cellular processes including glucose metabolism, cell proliferation and apoptosis.
AMP-activated protein kinase (AMPK): an enzyme expressed in several tissues that plays a pivotal role in cellular energy homeostasis.
Aryl hydrocarbon receptor nuclear translocator (ARNT): a basic helix-loop-helix protein required for the activity of the aryl hydrocarbon receptor (AhR). ARNT also dimerizes with hypoxia-inducible factor 1 (HIF1), and this heterodimer functions as a transcriptional regulator of the adaptive response to hypoxia.
db/db: a mouse model of T2D that lacks the leptin receptor.
Factor inhibiting HIF (FIH): an asparagine hydroxylase that inhibits the function of the HIF α-subunits. It requires iron, oxygen and 2-oxoglutarate for function.
Hypoxia-inducible factors (HIFs): heterodimeric transcription factors, with an oxygen-sensitive α subunit.
Non-alcoholic fatty liver disease (NAFLD): steatosis of the liver not caused by alcohol consumption.
PHDs: prolyl hydroxylase domain enzymes (PHDs) that hydroxylate (and thereby regulate) the HIF transcription factors.
TallyHo: mouse model of T2D with diabetes susceptibility loci on chromosomes 7, 13, 15, and 19.
Von Hippel–Lindau (VHL): a protein that forms part of an E3 ubiquitin ligase complex, and is responsible for ubiquitination and proteosomal degradation of HIFs.

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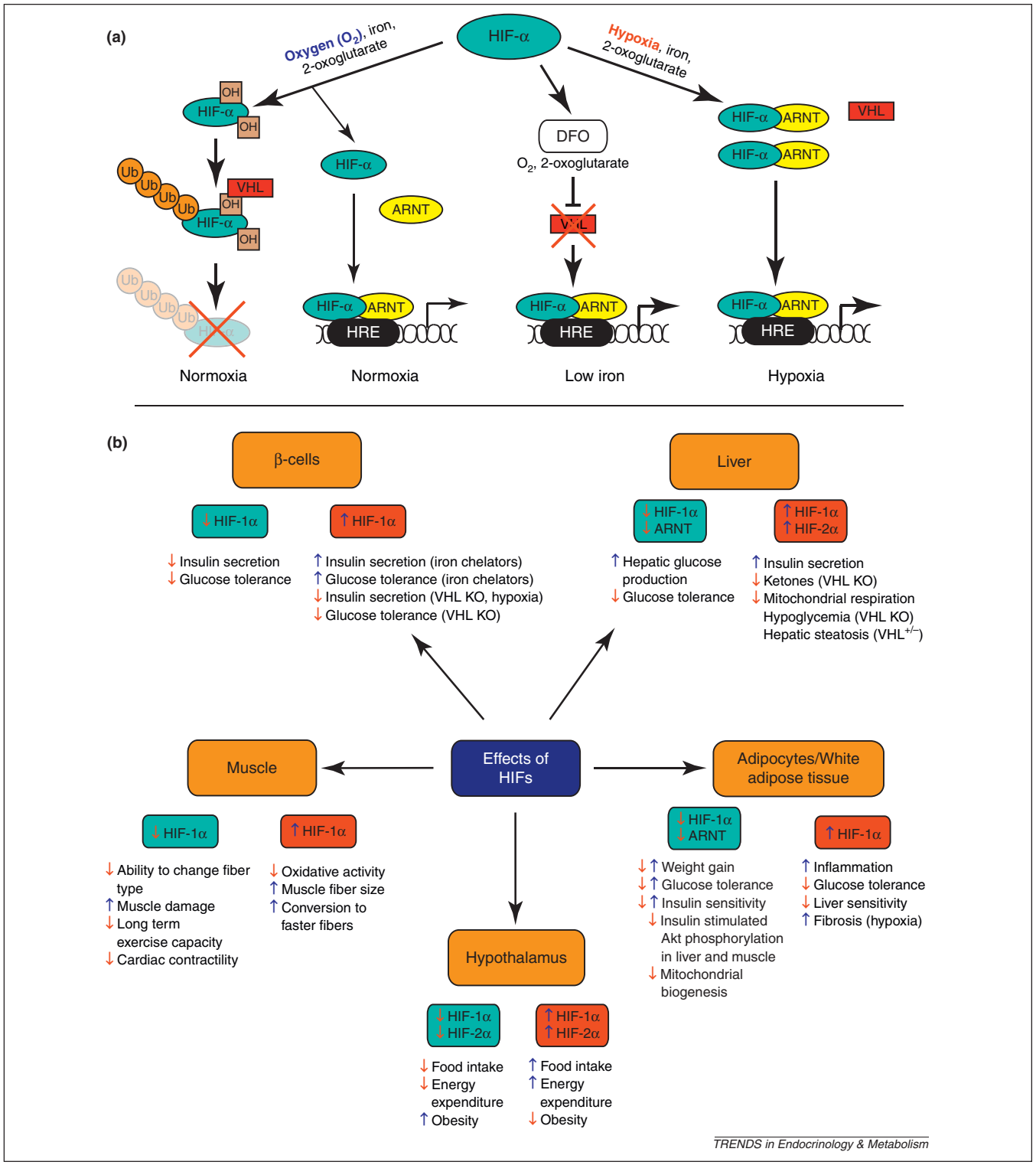

Figure 1. Regulation of HIF protein and the role of HIFs in various tissue types. **(a)** Under normoxic conditions HIF α subunits are hydroxylated, ubiquitinated, and targeted for proteosomal degradation. A small amount escapes degradation and binds to ARNT to regulate target genes. Under hypoxic conditions, HIF α subunits are unable to be hydroxylated by the oxygen-dependent hydroxylases and thus escape degradation. The iron chelator deferoxamine is able to stabilize HIF-1α because the hydroxylases also require iron to function. **(b)** HIFs are present in several tissue types and the effects of reducing or increasing HIFs from previous studies are summarized. Upward arrows indicate an increase, and downward a decrease. Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator; HIF, hypoxia inducible factor; VHL, von Hippel–Lindau; KO, knockout.

HIFs, diabetes and metabolic syndrome

Diabetes has become a problem of global proportions. The two major risk factors for T2D are insulin resistance and β-cell dysfunction. Recent research suggests that disturbances in HIF-1 signaling may play a detrimental role at several stages in diabetic pathogenesis, including the innate failure of β-cells to secrete sufficient insulin [12,13], insulin resistance [14,15], adipocyte dysfunction,

and inflammation [16–18]. For example, hyperglycemia interferes with protein stabilization, although the mechanisms are not fully understood [12,13]. Conversely, insulin and other growth factors may stabilize HIF-1α [14].

HIF-1 and β-cell function

β-Cell dysfunction leads to inadequate insulin secretion. In normal β-cells, rising glucose levels lead to its increased uptake and metabolism through glycolysis, the Krebs cycle,

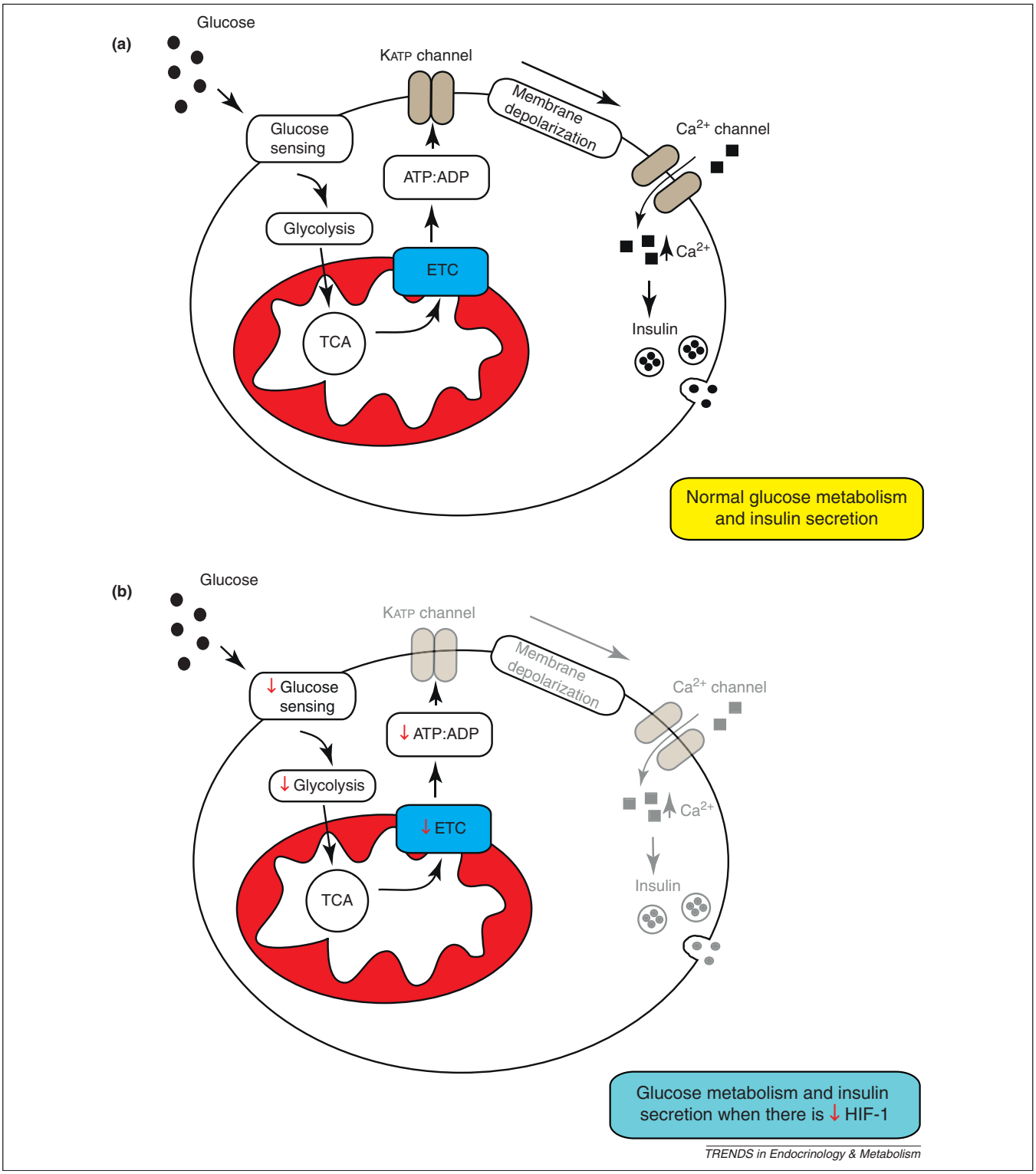

Figure 2. Hypoxia inducible factor (HIF) and pathways in β-cell dysfunction in diabetes. **(a)** Under normal conditions, glucose is taken up and broken down by glycolysis to provide substrates for the TCA cycle and the electron-transport chain. An increase in the ATP:ADP ratio closes the ATP-dependent potassium channels, leading to membrane depolarization, opening of the voltage-gated calcium channels and increase in intracellular calcium. This subsequently results in insulin exocytosis. **(b)** Decreased HIF-1 leads to decreases in genes associated with glucose sensing, glycolysis, and the electron-transport chain, ultimately leading to a decrease in ATP generation. A decrease in insulin secretion is a direct result of a decrease in the ATP:ADP ratio.

and then in the mitochondrial electron-transport chain (Figure 2a). Metabolism of glucose leads to generation of ATP from ADP. The ATP-sensitive potassium channels close, leading to membrane depolarization and insulin release. Thus, impaired glucose uptake can impair glucose-stimulated insulin release (GSIS).

Islets isolated from people with T2D have a 90% decrease in ARNT expression (mRNA) compared to glucose-tolerant donors [12]. Mice with β-cell-specific knockout of ARNT (β-ARNT) had reduced glucose tolerance and impaired *in vivo* and *in vitro* GSIS, and similar changes in gene expression to those seen in T2D islets, including decreased HNF4α, insulin receptor, aldolase, phosphofructokinase and others [12]. Cellular studies examining ARNT knockdown in three different β-cell culture models (MIN6, primary islets and 832/13) show consistent results, with significantly impaired insulin secretion, reduced glycolytic enzyme expression, and dysregulation of metabolic pathways [12,19]. These findings suggest that ARNT plays an important role in β-cell function. Another recent cellular study has identified carbohydrate-responsive element-binding protein (ChREBP) as a negative regulator of ARNT and has suggested that ChREBP-mediated repression of the HIF complex might contribute to glucotoxicity-induced β-cell dysfunction [20].

HIF-1α, the heterodimeric partner of ARNT, plays an important role in insulin secretion, because insulin secretion is impaired in mice with β-cell-specific knockout of HIF-1α and in MIN6 cells with siRNA knockdown of HIF-1α [21]. As with β-ARNT knockout mice, these mice had decreased expression of glycolytic genes including glucokinase and phosphofructokinase, key components of the β-cell glucose-sensing machinery [21] (Figure 2b).

Consistent with impaired glycolytic gene expression, β-HIF-1α null mice had severely reduced glucose-stimulated ATP generation and therefore impaired insulin release (Figure 2b). This provides a mechanism by which a transcription factor can regulate insulin secretion. Further linking these components is the observation that the uptake and metabolism of glucose by the electron-transport chain in a β-cell model was shown to deplete oxygen. Oxygen depletion induces HIF-1α and increased glycolysis [22].

VHL is required for HIF proteolysis, and thus deletion or inactivating mutations increase HIF protein. Interestingly, mice with β-cell deletion of VHL have markedly impaired insulin secretion and glucose intolerance [23–26]. Therefore, both depletion of ARNT or HIF-1α and excess of HIF-1α and HIF-2α (with are both increased with VHL deletion) impair β-cell function, suggesting an 'inverse-U' relation.

Because hydroxylation and concomitant degradation of HIFs requires iron, iron chelators induce modest increases in HIF protein levels. Use of iron chelators resulted in improved insulin secretion and normalized ARNT mRNA and downstream gene expression in islets from people with T2D [21]. The β-cell benefits of iron chelation are HIF-1α-dependent because iron chelators did not affect β-cell function in mice lacking HIF-1α in β-cells. Collectively, these data suggest that iron chelators might have therapeutic potential. Consistent with the inverse U-relation, deletion of FIH, which causes relatively modest increases in HIFs, also results in improved glucose tolerance in mice challenged with high-fat diet (HFD) [27].

The various effects in HIF-1 dyregulation of β-cells are shown in Figure 1, and the effects on β-cell glucose-sensing are shown in Figure 2b. Smaller increases such as those seen with iron chelation or FIH deletion are beneficial for β-cell function and survival [28–30]. Mice on a HFD with the addition of an iron chelator (deferasirox) have better glucose tolerance than mice on a HFD alone. Beneficial effects on β-cell function were not limited to mice because human islets treated with the iron chelator deferoxamine had improved glucose-stimulated insulin secretion and *ARNT* mRNA expression was restored [28]. Deferoxamine improves islet survival following the hypoxic insult of islet transplantation [29]. Mice were cured of diabetes with a lower number of transplanted islets when islets were pretreated with deferoxamine. Deferoxamine-treated islets had increased anti-apoptotic gene expression and decreased apoptosis [29].

The available evidence suggests that HIF-1α activity operates on a spectrum, with the two extremities of too little and too much HIF-1α causing detrimental effects on β-cell function. In this context it is interesting that whereas homozygous β-cell deletion of VHL is very clearly deleterious for β-cell function, heterozygous mutations in people with VHL syndrome do not appear to be associated with diabetes. In fact, there may be a decrease in diabetes risk after partial pancreatectomy in people with VHL syndrome (discussed in [28]). Our belief is that there is an optimal point for HIF-1α for β-cell function, with 'normal' probably being below that level of HIF-1α. Then, if HIF-1α is modestly increased, such as with iron chelation, β-cell function will improve.

Consistent with that concept, human islets from people with T2D have decreased expression of both HIF-1α and ARNT, and increasing HIF-1α with iron chelation improved human islet function and gene expression [28]. Iron chelators are currently approved for human use for treatment of iron overload and it will be of great interest to determine the effects in human diabetic patients in clinical trials.

HIFs and liver

In the liver, the highest oxygen concentrations are in the periportal regions and the lowest in the centrilobular area. Periportal hepatocytes specialize in oxidative metabolism and the synthesis of bile. Centrilobular cells have lower oxygen tension, higher HIF protein levels, take up more glucose, synthesize glutamine, and metabolize xenobiotics – functions related to the activity of ARNT and HIFs. Xenobiotic metabolism is regulated by ARNT plus Aryl hydrocarbon receptor (AhR) [31,32]. The liver is sensitive to hypoxia and to reperfusion injury: apneic episodes in people who have obstructive sleep apnea can induce acute elevation of liver enzymes [33,34].

Liver dysfunction is a key component of T2D and is both affected by and contributes to the condition [35–38]. Cardinal features include increased and inappropriate hepatic glucose production (HGP) [39,40] and reduced hepatic insulin sensitivity [41]. Non-alcoholic fatty liver diseases (NAFLD) are common in T2D and further exacerbate metabolic dysfunction [42–48].

Perturbations of the HIF proteins have been demonstrated to play a role in these processes in animal models. It has been shown that feeding mice with a high-fat/sucrose diet that causes fatty liver also causes upregulation of hepatic HIF-1α [49] via an unknown mechanism. However, HFD is a metabolic stress with well-established effects on glucose and energy homeostasis. When mice with a hepatocyte-specific HIF-1α deletion were fed this diet, they exhibited more severe impairment of glucose tolerance and peripheral insulin-resistance than control littermates [49].

Consistent with the decrease in ARNT mRNA in T2D islets, ARNT mRNA and protein are also reduced in human T2D livers [50]. Short-term hepatic ablation of ARNT in mice using adenovirus–*cre* injection increased HGP and impaired glucose tolerance [50]. Conversely, deletion of hepatocyte VHL substantially increased hepatic HIF-1α and HIF-2α proteins and caused life-threatening hypoglycemia and reduced ketones [51]. This phenotype was rescued by hepatic ARNT inactivation [51]. Hepatocyte VHL-null mice developed fatty liver, as did $VHL^{+/-}$ (heterozygous) mice [52]. Further studies reported that this effect is HIF-2α-dependent [53]. It is interesting that VHL knockout in β-cells results in impaired glucose tolerance whereas its specific knockout in the liver leads to hypoglycemia. The data from HIF-1α, ARNT and VHL deletion suggest that in the liver there is also an inverse-U relation, and both deletion and excess of HIF activity are deleterious. Similarly to in β-cells, this suggests that balanced HIF activity is crucial for optimal metabolic health.

As discussed in the β-cell section, the use of iron chelation in the form of deferoxamine may be beneficial to hepatic insulin sensitivity. In HepG2 hepatoma cells, deferoxamine upregulated the glucose transporter Glut1 and the insulin receptor. The latter was ARNT-dependent [54]. Rats treated with deferoxamine displayed improved insulin sensitivity with the upregulation of hepatic insulin receptor and Akt/PKB.

HIFs and muscle

Skeletal muscle is faced with the challenge of matching energy production with demand under widely-ranging circumstances, from resting to intense exercise. The ability of muscle to switch rapidly between aerobic and anaerobic energy production is an important characteristic [55,56]. HIF-1 plays a role in this dynamic process by regulating glycolytic and oxidative pathways of energy production, mitochondrial respiration and muscle fiber composition [55,57]. Its role is suggested by three observations: expression is higher in fast-twitch muscles that rely on glycolysis [58] and is upregulated during bursts of activity [57] and in chronic hypoxia [59].

Mice with muscle-specific HIF-1α deletion are initially able to exercise for longer due to reduced lactate accumulation [60]. Lactate accumulation causes muscle pain, hence decreased lactate associates with improved exercise tolerance. However, the mice lack the ability to change fiber-type between fast- and slow-twitch types, and have a decreased capillary:fiber ratio, making the muscle less able to adapt [55]. They develop muscle damage and fibrosis, eventually resulting in reduced exercise capacity [60]. Conversely, muscle HIF-1α overexpression in rats increased fiber size and conversion to faster fibers [57].

In humans, a HIF-1α polymorphism (Pro582Ser) is more commonly found in elite athletes versus controls, suggesting that it may improve physical performance and muscle function [61]. However, the mechanism is unknown. In a subset of skaters who had muscle biopsies, this polymorphism was associated with a predominance of fast-twitch fibers. Increased fiber size and fast-twitch fibers would improve strength and performance in tasks requiring speed, and therefore some have suggested that muscle HIF may one day constitute a form of doping [62]. The effects of iron chelators would be very interesting to examine in the context of human athletic performance and muscle function.

We have found two studies examining the direct role of muscle HIF-1 in glucose homeostasis. In the first study HIF-1α upregulated GLUT4 mRNA after 10 min of electrically-induced contraction of isolated soleus muscle. Thus HIF-1α might facilitate glucose transport following contraction [63]. Another study showed that insulin-dependent upregulation of glucose transporters was dependent on the HIF-1α/ARNT transcriptional complex [64]. Interestingly, basal and insulin-induced expression of Glut1, Glut3, aldolase, phosphoglycerate kinase, and VEGF were reduced in ARNT-defective cells.

The characteristic features of skeletal muscle in subjects with insulin resistance, namely reduced oxidative and increased glycolytic activities and altered fiber composition [65], may imply a potential regulatory role for HIF-1. However, direct assessment of glucose homeostasis in animal models will be needed to test this possibility.

HIFs, fat and inflammation

Since the discovery of leptin it has been recognized that adipose tissue represents a dynamic organ that produces adipokines and has roles in energy balance, glucose metabolism, inflammation and immunity [66,67]. Obesity is the strongest acquired risk factor for T2D: it gives a 10-fold higher risk in men and 30-fold higher risk in women [68,69].

Three recent reviews have discussed the various links between obesity and insulin resistance [67,70,71]. In insulin-resistant obesity there is adipose tissue hypoxia, and this appears to drive an inflammatory response [72,73]. In cultured adipocytes and preadipocytes, HIF-1α expression is upregulated by exposure to hypoxia or cobalt(II) chloride (a heavy metal poison which inhibits mitochondrial function and increases HIFs), and exposure increased levels of the inflammatory mediators IL-6 and monocyte migration inhibitory factor, and raised leptin and reduced adiponectin levels [74]. In obese mice, regional fat hypoxia colocalized with macrophage and T cell infiltration [75,76].

In obese humans, reduced oxygen pressures within abdominal fat associate with greater macrophage infiltration [77]. Collectively, these observations suggest that (i) adipose tissue hypoxia is deleterious and (ii) that defects in the adipocyte response to hypoxia may contribute to the pathogenesis of insulin resistance and diabetes. This concept was also suggested by a study in which gene expression analysis of cultured adipocytes taken from diabetic

db/*db* and *TallyHo* mice demonstrated that these cells were unable to respond efficiently to hypoxia [78].

Compelling evidence that HIF-1 plays a crucial role in adipocyte function emerges from genetically engineered models. In mice overexpressing constitutively active HIF-1α in white adipose tissue (WAT), inflammation, glucose intolerance and decreased liver sensitivity were observed both on normal chow and on a HFD [16]. Also, as seen in β-cells, constitutively active, unregulatable HIF is deleterious [28].

Studies examining adipocyte-specific HIF-1α and adipocyte-specific ARNT knockout mice report the protection of these mice from the consequences of HFD. Specifically, these mice were resistant to weight gain, and had substantially better glucose tolerance and insulin sensitivity [17,18]. Increased insulin-stimulated Akt phosphorylation was seen in WAT, liver and muscle in adipocyte-specific HIF-1α knockout mice [17], suggesting crosstalk between adipose tissue and other sites. These mice also displayed central effects with an increase in core temperature and energy expenditure [17,18]. Adipocyte-specific ARNT knockout mice were lean, had smaller adipocytes, and were protected from age-related effects on glucose homeostasis [79]. In these studies, adipocyte specific ARNT and HIF-1α mice were created using the *aP2 (Fabp4)–cre* strain. The presence of *aP2–cre* causes selective deletion of a 'floxed' gene in cells expressing aP2 (adipocyte protein 2/fatty acid binding protein 4) – which include macrophages and potentially some cells in the central nervous system (CNS) [80]. Deletion in macrophages may contribute to the fascinating metabolic phenotype because macrophage accumulation and activity are linked to obesity [81].

By contrast, another mouse model of adipocyte-specific HIF-1α inhibition resulted in entirely different effects [82]. In response to HFD, these mice gained more weight, had worse glucose tolerance and insulin sensitivity, and impaired mitochondrial biogenesis. The reason for the opposite responses is unclear. In these mice a dominant negative mutant of human HIF-1α lacking the DNA-binding domain was overexpressed in adipocytes, and there was also substantially increased expression in brown adipose tissue (BAT). In contrast to 'normal' adipose tissue, the primary function of BAT is not to store energy, but to consume energy in thermogenesis. Thus, some of the effects reported in this paper may relate to preferentially decreased HIF-1α function in BAT. Moreover, *aP2–cre* also deletes in BAT, and therefore in the genetic deletion models discussed above there would also have been HIF-1α deletion in BAT.

Deletion of VHL from adipocytes, also using *aP2–cre* mice, caused brain hemorrhages and lethality, reflecting *aP2–cre* expression in the embryonic CNS [80]. This raises the additional possibility that some of the beneficial effects seen in *aP2–cre* ARNT and *aP2–Hif1a* mice may relate to CNS effects.

The findings from another mouse model further complicate the interpretation. Mice lacking FIH have greater HIF activity in fat and are protected from the effects of HFD, with decreased weight gain, decreased fat accumulation, and improved insulin sensitivity [30]. Many of the effects in the FIH null mice were recapitulated by selective deletion in the CNS [30].

An intriguing finding of these papers is that peripheral knockout of HIF-1α can alter central control of metabolism. Changes in the metabolic rate, as assessed by indirect calorimetry (that measures heat production based on

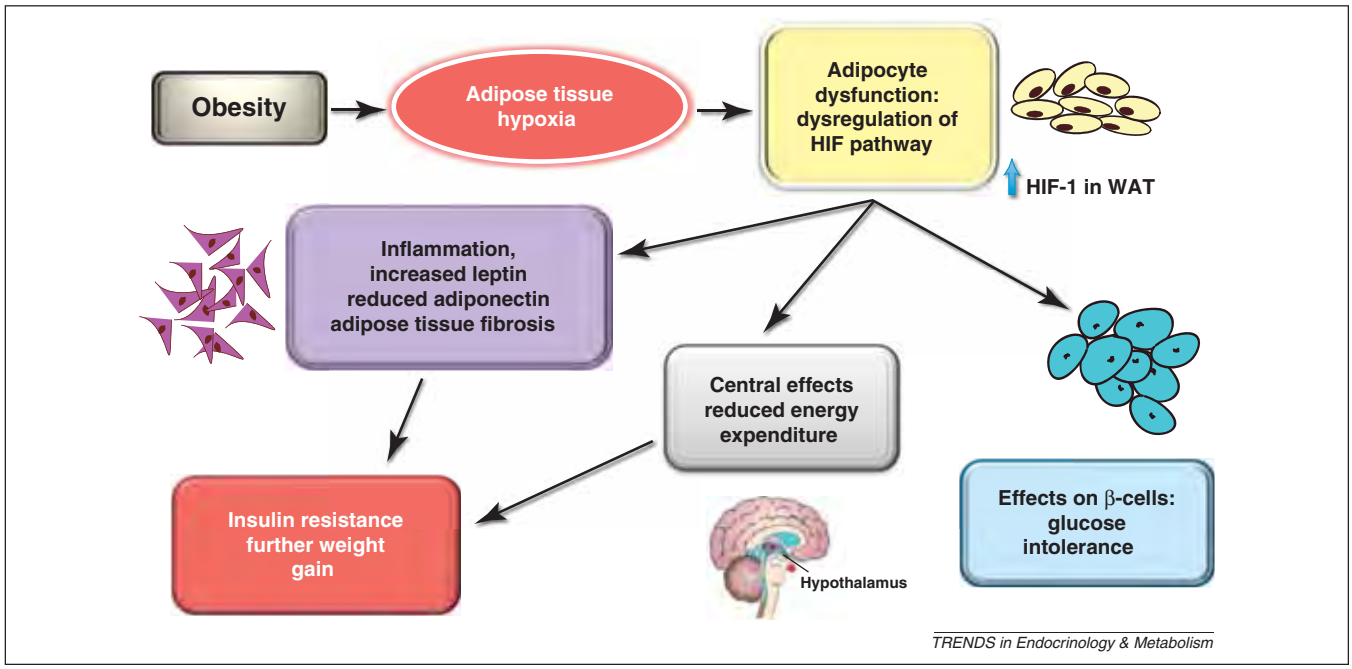

Figure 3. Roles of HIF-1α in adipocyte dysfunction and impaired glucose homeostasis. In obese individuals, hypoxia of adipose tissue leads to a chain of local events set off by dyregulation of the HIF-1 pathway in adipocytes, including inflammation and macrophage infiltration. More distant effects include disturbances in β-cell function and the central control of metabolism, all of which ultimately lead to glucose intolerance, insulin resistance, and further weight gain. The main evidence for this model comes from transgenic mouse studies; whether this takes place in humans under typical pathophysiological conditions is not yet clear. Abbreviations: HIF, hypoxia inducible factor; WAT, white adipose tissue.

oxygen consumption), and changes in core body temperature in adipocyte-specific models, support this possibility [20,21,81]. A recent study focusing on the hypothalamus examined this issue [83]. Neurons in the mediobasal hypothalamus express HIFs, particularly HIF-2α, and this protein was upregulated in response to glucose, suggesting that HIF-2α regulates hypothalamic glucose-sensing. HIF activation upregulated expression of pro-opiomelanocortin, an anorexigenic neuropeptide that is essential in the control of body weight. Conditional loss-of-function of HIF signaling in pro-opiomelanocortin hypothalamic neurons exacerbated dietary obesity. Delivery of HIF-2α/ARNT or HIF-1α/ARNT via lentiviral coexpression in the mediobasal hypothalamus protected these mice from the effects of a HFD. Favorable effects of both HIF-1α and -2α were attributed to reduced *ad libitum* food intake [83].

How do we incorporate these findings into a unifying explanation of the role of HIFs in adipocyte function, energy regulation, and glucose homeostasis? First, the local upregulation of HIF-1α in adipocytes in obese individuals with hypoxic WAT is associated with inflammation and peripheral insulin-resistance. This is corroborated by similar findings from a mouse model which overexpresses HIF-1α in adipocytes. On the other hand, deletion of HIF-1α in adipocytes appears beneficial, conferring protection from disturbances of glucose homeostasis and energy regulation following a HFD. These concepts are depicted in Figure 3. Excess HIF-1α, induced by hypoxia, is deleterious, and deficit appears to be beneficial. However, expression of a dominant negative form of HIF-1α adipocytes is deleterious [82]. Second, whole-body FIH deletion, which modestly increases HIF, is beneficial. Together, this suggests that in adipocytes the relation is more complex than the apparent inverse-U-shape in other tissues. It remains unclear why the relation in adipose tissue is different.

HIF and diabetes complications

The complications posed by diabetes lead to significant morbidity and mortality and a substantial financial burden to the community. Hypoxia is a key feature of these complications whether they occur at a macrovascular (i.e., coronary artery, peripheral and cerebral vascular diseases) or microvascular level (i.e., neuropathy, retinopathy, nephropathy).

Mechanisms by which hyperglycemia results in the degradation of HIF-1α have been described [84], possibly linking the increase in myocardial infarct size amongst rats exposed to hyperglycemia with the reported reduction in HIF-1α [85]. More directly, overexpression of HIF-1α leads to improvement of myocardial circulation and inhibition of cardiac hypertrophy and fibrosis following myocardial injury in diabetic mice [86]. These effects probably take place at the basic level of endothelial function and thus oxygen and nutrient supply, as suggested by the upregulation of VEGF by HIF-1α in these mice.

Biopsies taken from the foot ulcers of human subjects with diabetes displayed significantly lower HIF-1α levels than biopsies taken from venous ulcers, suggesting a role for hyperglycemia rather than hypoxia alone in diabetic peripheral vascular disease [84]. Young *db*/*db* (obese) mice with large wounds display reduced HIF-1α production, and genetic upregulation of HIF-1α in these mice accelerated wound-healing and angiogenesis [87]. In another study, the topical application of deferoxamine improved healing of skin wounds in *db*/*db* mice and systemic administration improved surgical skin-flap survival in streptozocin-induced diabetic mice [88]. Further research is needed to clarify the role of HIF-1 and its potential as a therapeutic target in the management of these important conditions.

Box 1. Outstanding questions

- How does a local imbalance in HIF-1 levels facilitate crosstalk between remote organ systems? For example, adipocyte-specific knockout mice display increased body temperature and energy expenditure, usually under the control of the hypothalamus.
- A better understanding of the complexities of HIF-1 regulation may help to explain some of the discordant findings of various mouse models with unregulated, increased HIF-1 activity. Also, by contrast, when there is whole-body unregulated HIF-1, as in FIH mice, why is this beneficial?
- What are the effects on function of various metabolic tissues in people with VHL syndrome: is their rate of diabetes altered? Do they have an increased or decreased risk of fatty liver?
- What are the effects of mutations in the equivalents of FIH and PHDs in humans? Again, do these people have a decreased risk of diabetes? Are they protected from obesity as are the FIH mice?
- How can we apply this evidence-base to the treatment of T2D and metabolic syndrome?

Concluding remarks

Collectively, the literature indicates important roles for the HIFs in many tissues, with tissue crosstalk. Several transgenic animal studies have reported effects of organ-specific ARNT and HIF knockouts, raising the suggestion that HIF-1-mediated crosstalk plays a role in the pathogenesis of diabetes. Indeed, T2D is a disorder of seemingly disparate phenomena, including defective β-cell function, impaired insulin signaling within muscle, and increased HGP. Each of these individual features can be induced or repressed by manipulating ARNT and/or HIFs. Given the known alterations in human tissues we speculate that ARNT and HIFs may play a central role in these processes. It is interesting to speculate whether HIF-related perturbations in glucose homeostasis point to an underlying problem in hypoxia-sensing or are hypoxia-independent. There are also a number of outstanding questions posed by this body of evidence (Box 1). In adipose tissue the hypoxia model plausibly links together HIF-related maladaptive responses to tissue hypoxia, with subsequent inflammation and peripheral insulin resistance. Likewise, vascular complications in diabetes are characterized by tissue hypoxia, with plausible evidence for a contributory role in the dyregulation in the HIF-1 pathway. For the β-cell and liver the distinction is less clear. It is likely that hypoxia-independent factors play a role in HIF dyregulation in β-cells.

Interpreting these findings in a clinical context is challenging. Tissue-specific manipulation of the HIF pathway is clearly difficult to translate to the whole human organism. However, overall, in most tissues, especially fat, hypoxia is obviously deleterious for function. For β-cell function, modest hypoxia, similar to the exposure *in vivo* (5% oxygen), is not deleterious, but function is clearly impaired at lower oxygen levels. Interestingly, in muscle,

intermittent hypoxia induced by exercise is beneficial, and induces helpful adaptive responses, but chronic hypoxia is deleterious.

Despite its limitations, this body of preclinical data supports a vital role for HIF-1 in the regulation in mice of glucose homeostasis, adipocyte function and the central regulatory control of metabolism. In humans, significant alterations in the expression of HIF-1α and/or ARNT in the islets and liver cells of those with diabetes suggests that HIF-1 pathway may also play similar roles in glucose homeostasis to those demonstrated in mice. Together, the body of animal research and the human data suggest that modest stimulation of the HIF pathway may present a viable therapeutic strategy in the management of T2D and the metabolic syndrome.

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Hypoxia-inducible factor-1α regulates β cell function in mouse and human islets

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Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that regulates cellular stress responses. While the levels of HIF-1α protein are tightly regulated, recent studies suggest that it can be active under normoxic conditions. We hypothesized that HIF-1α is required for normal β cell function and reserve and that dysregulation may contribute to the pathogenesis of type 2 diabetes (T2D). Here we show that HIF-1α protein is present at low levels in mouse and human normoxic β cells and islets. Decreased levels of HIF-1α impaired glucose-stimulated ATP generation and β cell function. C57BL/6 mice with β cell–specific *Hif1a* disruption (referred to herein as β-*Hif1a*-null mice) exhibited glucose intolerance, β cell dysfunction, and developed severe glucose intolerance on a high-fat diet. Increasing HIF-1α levels by inhibiting its degradation through iron chelation markedly improved insulin secretion and glucose tolerance in control mice fed a high-fat diet but not in β-*Hif1a*-null mice. Increasing HIF-1α levels markedly increased expression of *ARNT* and other genes in human T2D islets and improved their function. Further analysis indicated that HIF-1α was bound to the *Arnt* promoter in a mouse β cell line, suggesting direct regulation. Taken together, these findings suggest an important role for HIF-1α in β cell reserve and regulation of ARNT expression and demonstrate that HIF-1α is a potential therapeutic target for the β cell dysfunction of T2D.

Introduction

The transcription factor HIF-1α is important for a range of functions, including cellular responses to hypoxia and other stressors, angiogenesis, and fetal development (1–6). It has strong antiapoptotic effects (7–11) and is implicated in the pathogenesis of cardiovascular diseases and some cancers (12–20).

HIF-1α is a member of the bHLH-PAS family (reviewed in refs. 2, 18, 21) and functions as an obligate dimer with other family members, including aryl hydrocarbon receptor (AhR) nuclear translocator (ARNT). We previously reported that ARNT was decreased in islets isolated from patients with type 2 diabetes (T2D) and that decreasing ARNT in Min6 cells or disrupting it in mouse β cells caused changes in gene expression and glucose-stimulated insulin secretion (GSIS) similar to those seen in islets isolated from humans with T2D (22). Recently, we reported a loss of ARNT expression in the livers of people with T2D, affecting dysregulation of gluconeogenesis (23). Though the specific ARNT partner which is important for its actions in β cells (or liver) is not known, candidates include AhR, HIF-1α, HIF-2α, HIF-3α, and circadian rhythm molecules, e.g., BMAL.

Because of its role in the regulation of glycolysis and other biological processes in other tissues (24, 25), we hypothesized that (a) HIF-1α might be the important partner for ARNT in β cells, (b) that decreasing HIF-1α would impair β cell reserve and thus lead to diabetes under conditions of β cell stress, and (c) that increasing HIF-1α in a nontoxic way would improve β cell function.

Consistent with its role in regulating a number of important biological processes, HIF-1α protein is tightly regulated (reviewed in refs. 2, 17, 19, 21, 25, 26). In the basal state, it is hydroxylated on proline residues and becomes competent to associate with von Hippel-Lindau (VHL) protein, leading to ubiquitination and rapid proteolysis, giving a half-life of minutes (19, 27, 28). Oxygen, iron, and 2-oxoglutarate are required for hydroxylation (29–32). Thus, hypoxia inhibits degradation, leading to a rapid increase. In addition, HIF-1α protein can be increased by genetic inactivation of VHL or the hydroxylases, treatment with heavy metals such as cobalt chloride, or iron chelation with deferoxamine (DFO) or deferasirox (DFS) (20, 29). An additional layer of regulation is added by asparaginyl-hydroxylation, which inhibits association with transcriptional cofactors, including p300 (21).

Until recently, it was thought that HIF-1α did not function under normoxic conditions. However, the presence of HIF-1α protein in brain, kidney, liver, embryonic stem cells, trophoblastic cells, and others (5, 6, 33) is now recognized. It is stabilized by inflammation,

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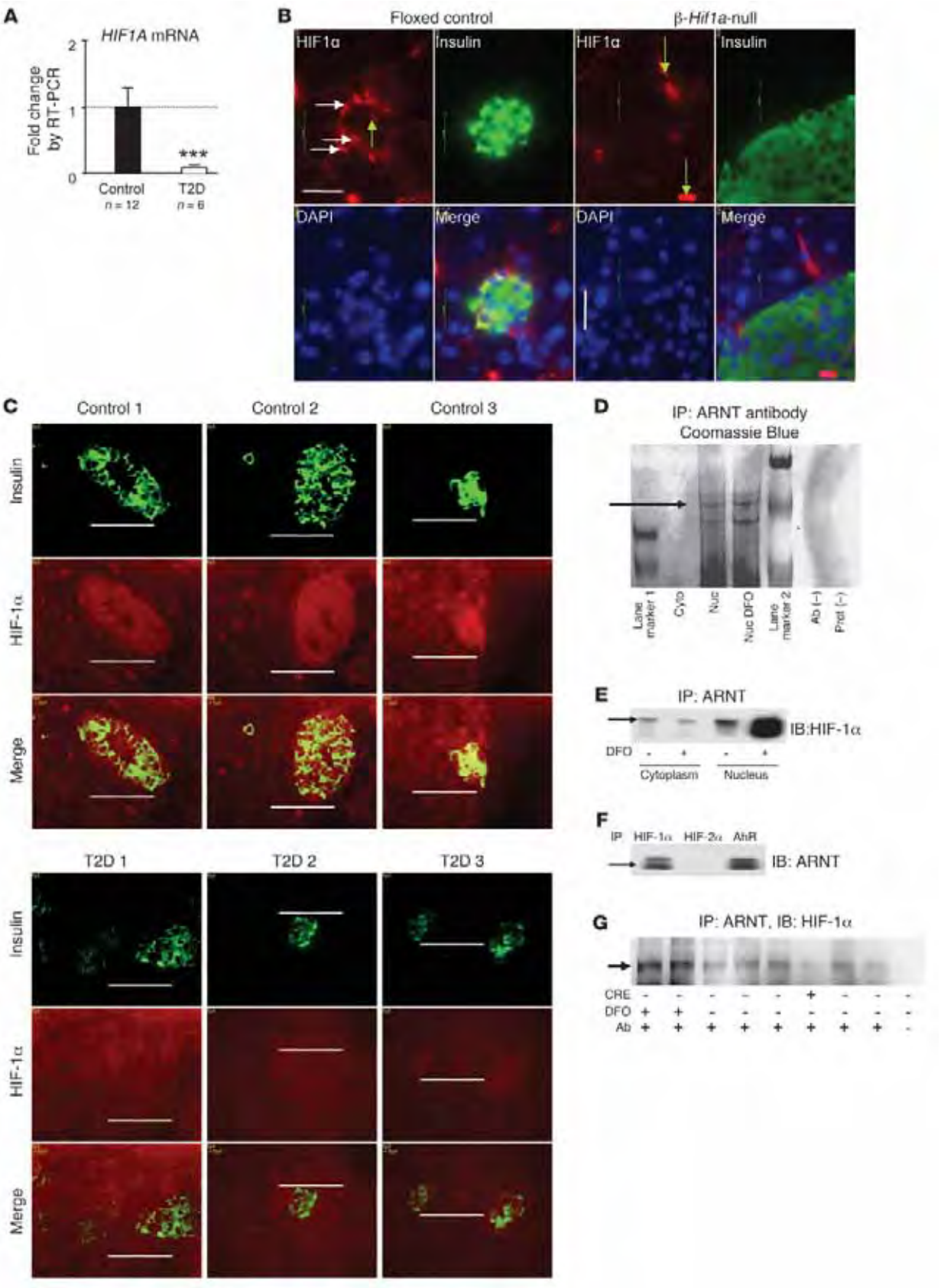
A
HIF1A mRNA
Fold change by RT-PCR
2
1
0
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Control
n = 12
T2D
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B
Floxed control
β-Hif1a-null
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IP: ARNT antibody
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Figure 1
HIF-1α is present in normoxic β cells, associates with ARNT, and is decreased in T2D. (**A**) *HIF1A* mRNA was decreased in islets of people with T2D ($n = 6$) compared with people with normal glucose tolerance ($n = 12$). ***$P < 0.001$. (**B**) HIF-1α protein was present in β cells in floxed control mice (horizontal arrows) but was decreased in β-*Hif1a*-null mice. In both genotypes, HIF-1α was present in blood vessels (vertical arrows). Scale bar: 20 μm. (**C**) HIF-1α protein was higher than background in people with normal glucose tolerance (top panels) but was decreased in T2D pancreata (bottom panels). Scale bar: 50 μm. (**D**) HIF-1α protein (arrow) was associated with ARNT by affinity purification. Cyto, cytoplasm; Nuc, nucleus; Prot, protein. (**E**) HIF-1α protein associated with ARNT in the basal state in Min6 cells, and nuclear HIF-1α increased with DFO. (**F**) ARNT protein associated with HIF-1α by coimmunoprecipitation. (**G**) HIF-1α protein was increased by DFO treatment of isolated mouse islets and was decreased in islets from a β-*Hif1a*-null mouse (Cre$^+$).

TGF, PDGF, EGF, and IL-1β (20, 34, 35) and by increased levels of ROS (36–38). Of potential relevance to β cells, insulin increases HIF-1α activity in liver, muscle, breast carcinoma, prostate carcinoma, and retinal epithelial-derived cells (39–42). PI3K-Akt pathway activation is required for the insulin-induced increase (43).

The role of HIF-1α in islets is not fully understood. Pancreatic islets are normally exposed to relatively low oxygen tension (20–37 mmHg) (44, 45) and to locally secreted insulin. These factors suggest a possible role for HIF-1α in islets and the possibility for decreased HIF-1α in the setting of insulin resistance.

This study found that targeted disruption of HIF-1α in β cells of C57BL/6 mice (referred to herein as β-*Hif1a*-null mice) led to glucose intolerance with impaired ATP generation and GSIS in isolated islets. Conversely, increasing HIF-1α using iron chelation with DFO or DFS caused significant changes in gene expression, which differed from severe hypoxia or VHL deletion (46–48). DFS significantly improved glucose tolerance in mice receiving a high-fat diet (HFD) but had no effect in β-*Hif1a*-null mice, demonstrating that β cell HIF-1α was required for its effect. Importantly, DFO treatment of T2D islets normalized expression of *ARNT* and downstream genes and improved GSIS. HIF-1α bound to the ARNT promoter, as revealed by ChIP, and increasing HIF-1α levels increased *ARNT* expression. Taken together, these findings suggest that decreased HIF-1α levels impair β cell reserve and that iron chelation, which increases HIF-1α activity in β cells, may be a therapeutic strategy for the treatment of human T2D.

Results

HIF-1α was present at low levels in islets and was decreased in humans with T2D. HIF1α levels were assessed using real-time PCR in isolated human T2D and control islets and using immunohistochemistry of pancreatic sections collected during partial pancreatectomy. *HIF1A* mRNA was decreased by 90% in T2D islets ($P < 0.0001$; Figure 1A). Immunohistochemistry revealed HIF-1α protein in some normal murine β cells (Figure 1B, horizontal arrows), and staining was present in blood vessels (Figure 1B, vertical arrows). In β-*Hif1a*-null islets, staining was present in vascular cells (Figure 1B, vertical arrows) but not β cells, demonstrating antibody specificity. In human partial pancreatectomy tissue, there was mild, diffuse HIF-1α staining, consistent with the operative procedure in which vessels are ligated prior to tissue removal, thus inducing hypoxia. Subjects with normal glucose tolerance showed more intense HIF-1α staining in islets than in the acinar pancreas (Figure 1C). In contrast, in T2D islets, HIF-1α staining was not higher than that in the acinar pancreas (Figure 1C).

HIF-1α associated with ARNT. To determine whether the HIF-1α in β cells and islets was associated with ARNT and therefore potentially transcriptionally active in the basal (nonhypoxic) state, we used ARNT affinity purification and tandem MALDI-TOF mass spectrometry. The Coomassie-stained gel showed a band at the appropriate size for HIF-1α in the untreated nuclear fraction and in the DFO-treated positive control (Figure 1D, black arrow). Mass spectrometry revealed 4 peptide sequences derived from HIF-1α. HIF-2α was also associated with ARNT but only in DFO-treated cells. AhR protein was not detected.

To confirm that ARNT was bound to HIF-1α, we performed coimmunoprecipitation studies in Min6 cells. Immunoprecipitation with ARNT antibodies revealed low levels of associated HIF-1α in the cytoplasm and nucleus, under normoxic conditions (Figure 1E). As expected, DFO treatment increased nuclear HIF-1α. The converse was also true; immunoprecipitation with HIF-1α antibodies purified ARNT, under normoxic conditions (Figure 1F, left lane). AhR antibodies were able to "pull-down" ARNT from whole-cell lysates (Figure 1F, right lane). However, without exogenous ligand, AhR antibodies did not purify detectable amounts of ARNT from nuclear extracts, suggesting that it was not functionally active (data not shown). As shown in Figure 1G, ARNT immunoprecipitation and HIF-1α immunoblotting was performed in whole-cell lysates from primary mouse islets. Lanes 1 and 2 are islets from mice treated with DFO and then cultured in DFO. Lanes 3–8 were islets cultured in normal media. The Cre$^+$ lane shows islets from a β-*Hif1a*-null mouse in which there was decreased HIF-1α.

β cell–specific Hif1α-null mice have impaired β cell function. Using the Cre-lox system, with Cre under control of the rat insulin promoter (RIP-Cre), and mice with a floxed *Hif1a* gene (floxed controls) (49), we generated β-*Hif1a*-null mice. HIF-1α immunostaining is shown in Figure 1B.

Importantly, in our colony, RIP-Cre mice have normal glucose tolerance (Figure 2A). β-*Hif1a*-null mice were fertile and did not differ in size or weight (data not shown). Fasting glucose did not differ among groups (Figure 2A); however, levels after glucose loading were significantly higher in β-*Hif1a*-null mice than in floxed control mice or in RIP-Cre mice. Disruption of HIF-1α in β cells did not alter fasting insulin. However, as shown in Figure 2B, β-*Hif1a*-null mice had significantly impaired first-phase GSIS. Consistent with these in vivo effects, islets isolated from β-*Hif1a*-null mice had impaired GSIS (>80% reduction at 3.3 and 11 mmol/l glucose; Figure 2C). The difference was not statistically significant at 22 mmol/l.

Gene expression was assessed in isolated islets using real-time PCR. In β-*Hif1a*-null islets, there was a more than 40% decrease in *Glut2*, glucokinase (*Gck*), glucose-6-phosphoisomerase (*G6pi*), phosphofructokinase (*Pfk*), hepatocyte nuclear factor 4α (*Hnf4a*), and others (Figure 2D). As expected, control islets had higher ATP content after exposure to high glucose (60% increase; Figure 2E). Islets from β-*Hif1a*-null mice had significantly lower basal ATP levels (60% decrease) and the increase in ATP levels with 25 mmol/l glucose was severely blunted. This was despite similar islet insulin content (Figure 2F) and nonsignificant, higher β cell content in β-*Hif1a*-null mice (Figure 2G). No differences were observed for CD31 staining, indicating similar islet vascularity (data not shown).

Hif1a knockdown in Min6 cells impaired β cell function. To confirm the β-*Hif1a*-null mice results in a β cell line, we used RNAi in Min6 cells. RNAi achieved approximately 70% knockdown of *Hif1a*

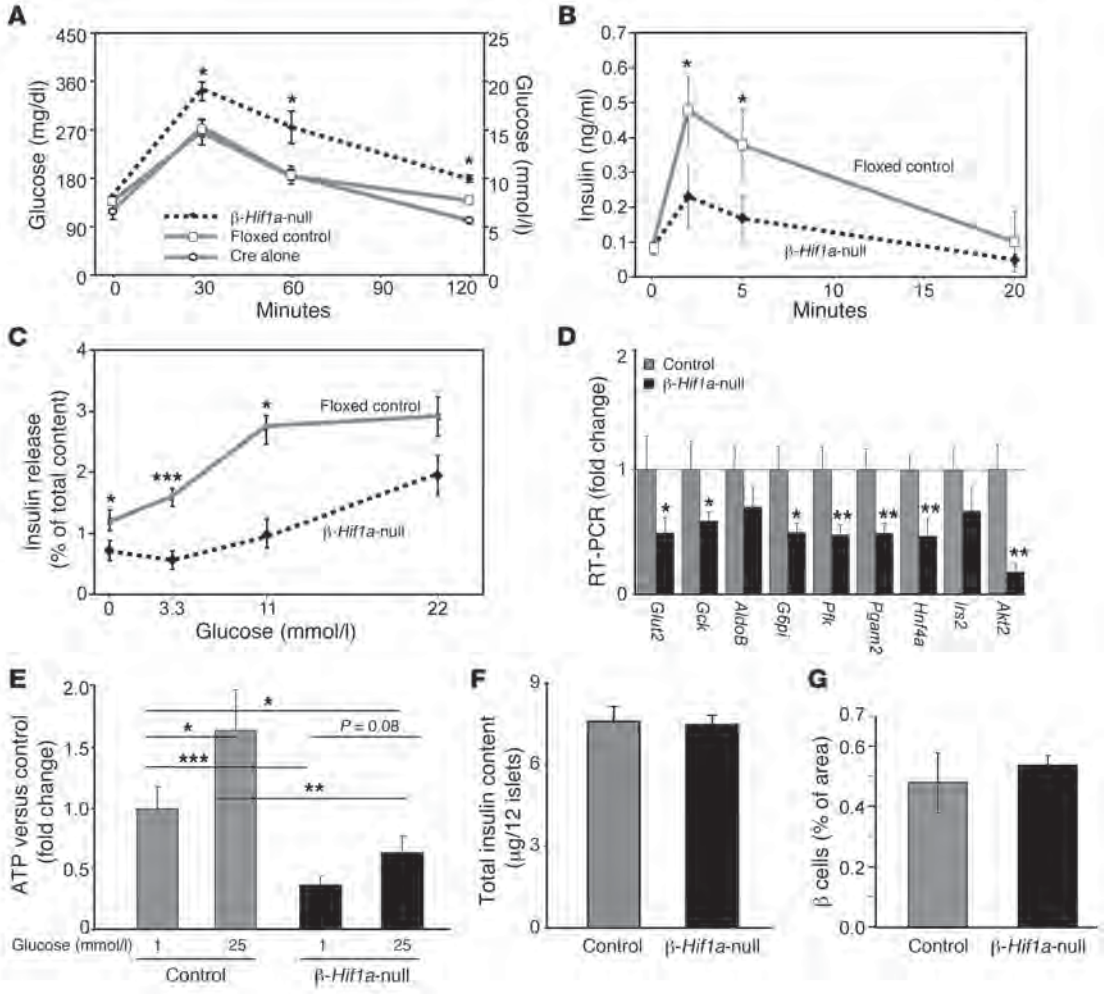


Figure 2

β cell deletion of Hif1a in mice causes glucose intolerance, impaired gene expression, ATP generation, and insulin secretion. (**A**) β-*Hif1a*-null mice were glucose intolerant compared with either floxed controls or RIP-Cre alone mice. *n* = 15, 15, and 12, respectively. (**B**) GSIS was decreased in β-*Hif1a*-null mice. (**C**) GSIS was decreased in isolated β-*Hif1a*-null islets. (**D**) Expression of several genes was decreased in β-*Hif1a*-null islets. (**E**) ATP concentrations were significantly decreased in β-*Hif1a*-null islets at both basal and high glucose levels. (**F**) Insulin content did not differ between floxed control and β-*Hif1a*-null islets. (**G**) β cell mass did not differ between groups. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

mRNA (Figure 3A). This was accompanied by markedly impaired GSIS (Figure 3B). There was a milder (approximately 25%) impairment in KCl-stimulated insulin release (Figure 3B, right). In separate experiments examining combined RNAi treatments, slightly more severe impairment occurred with *Arnt* RNAi and with *Hif1a* plus *Hif2a* plus *AhR* knockdown, suggesting small additional roles for Hif-2α and AhR (Figure 3C). KCl-stimulated insulin secretion was again impaired by approximately 25% (Figure 3C, right), suggesting a partially glucose-specific effect.

Decreased expression of glucose transporter and glycolytic mRNAs was found, including *Gck*, *Glut2*, *G6pi*, Aldolase (*AldoB*), and *Pfk* (40%–60%; Figure 3, D and E). These changes were similar to those for β-*Hif1a*-null islets (Figure 2E). As shown in Figure 3F, *Hif1a* knockdown impaired ATP generation basally (25% decrease) and following glucose stimulation (only 90% of basal control).

Increasing HIF-1α improved glucose tolerance in HFD-fed C57BL/6 mice. As noted above, β-*Hif1a*-null mice exhibited mild glucose intolerance, with preserved fasting glucose (Figure 2A). In a separate cohort of mice (Figure 4A), we performed glucose tolerance testing (GTT) and replicated the finding of impaired glucose tolerance, with preserved fasting levels ($P = 0.007$, ANOVA for repeated measures). For β-*Hif1a*-null mice, weight was 24.7 ± 0.6 g, and for controls, weight was 25.2 ± 0.6 g.

To examine β cell compensation in the setting of insulin resistance, we placed mice on a HFD (45% of calories from fat) for 3 weeks. Weight increased to 29.4 ± 0.9 g for β-*Hif1a*-null mice and to 29.0 ± 0.8 g for controls. GTTs deteriorated in floxed controls (Figure 4B, dotted line) but more severely in β-*Hif1a*-null mice (Figure 4C, dotted line). AUCs are shown in Figure 4D ($P = 0.047$ for floxed control HFD-fed versus β-*Hif1a*-null HFD-fed mice).

Following 3 weeks of HFD, all mice were changed to HFD admixed with DFS (HFD plus DFS) to increase HIF-1α. After 3 weeks, weight was 29.0 ± 0.8 g in β-*Hif1a*-null mice versus 29.0 ± 0.8 g in controls. As shown in Figure 4C, despite continuing HFD, floxed controls had highly significantly improved glucose tolerance. In contrast, there was no improvement in β-*Hif1a*-null mice, demonstrating that

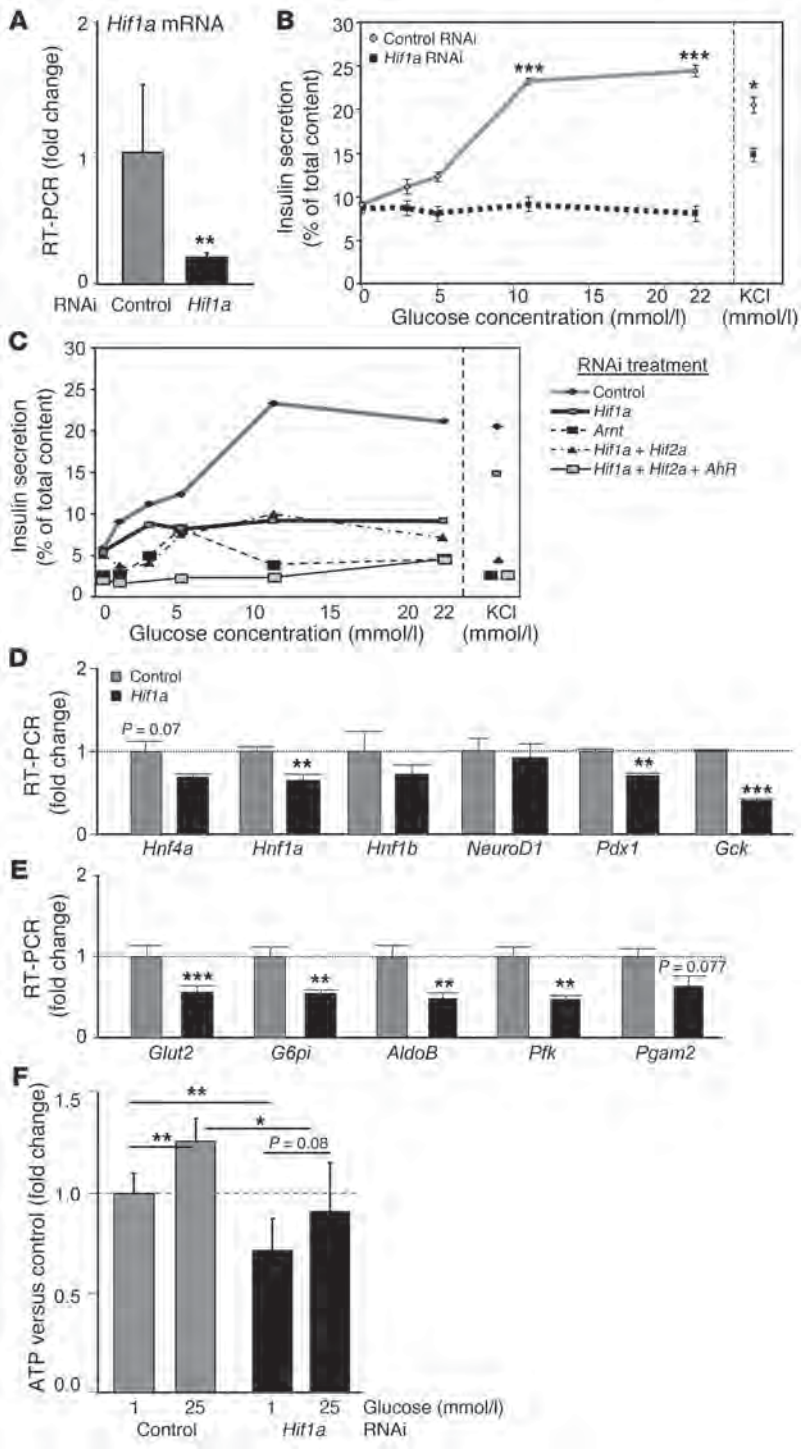


Figure 3
Decreasing *Hif1a* by RNAi-impaired β cell function, gene expression, and ATP generation. (**A**) RNAi decreased *Hif1a* mRNA. (**B**) *Hif1a* RNAi decreased GSIS in Min6 cells and caused a small decrease in KCl-stimulated insulin release. (**C**) Combination RNAi treatment caused slightly more severe impairment in insulin release. (**D**) *Hif1a* RNAi decreased expression of genes from the MODY family and (**E**) glucose-uptake and glycolysis genes. (**F**) *Hif1a* RNAi decreased basal and glucose-stimulated ATP concentrations. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

β cell HIF-1α was required for DFS to improve glucose tolerance. AUCs are shown in Figure 4D. Interestingly, β-*Hif1a*-null mice had 69% greater β cell mass than controls at the end of the study (Figure 4E), despite worse glucose tolerance, suggesting attempted and unsuccessful β cell compensation.

There was some 4-hydroxynonenal staining in HFD-fed mice, consistent with increased ROS, but it was without obvious differences between genotypes (data not shown). Similarly, β cell apoptosis did not differ, as assessed by cleaved caspase-3 staining. Apoptosis rates were less than 1% in both floxed control and β-*Hif1a*-null groups with this short-term treatment (data not shown).

We confirmed the effects of DFS on HFD-induced glucose intolerance in a separate cohort of normal (not genetically modified) C57BL/6 mice fed HFD or HFD plus DFS for 26 weeks. Glucose tolerance was significantly better in mice receiving HFD plus DFS (Figure 4F, solid line) compared with mice receiving HFD alone (Figure 4F, dotted line). In this 26 week experiment, weight was lower in the HFD plus DFS–fed group (HFD-fed, 38.8 ± 4.2 g versus HFD plus DFS–fed, 33.0 ± 3.2 g; $P = 0.037$), which may have contributed to the difference in GTTs. However, weight and fasting glucose were not significantly correlated ($P > 0.13$; Figure 4G) and neither were weight and AUC of GTTs (data not shown). Multivariate regression using weight and DFS as univariate predictors showed that only DFS independently predicted fasting glucose ($P = 0.010$ for DFS, $P = 0.811$ for weight) and AUC of GTTs ($P = 0.039$ for DFS, $P = 0.433$ for weight). Mice were not anemic (hemoglobin, 120 ± 7 mg/dl in DFS mice versus 124 ± 3 mg/dl in controls; $P > 0.6$). Insulin tolerance testing (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI35846DS1) showed that the HFD plus DFS–fed mice had similar percentages of decreases in blood glucose after insulin administration.

Increasing HIF-1α improved glucose tolerance in HFD-fed Balb/c mice. Because the above experiments were all performed in C57BL/6 mice, we sought to confirm the effects in another mouse strain. Balb/c mice were weighed (20.8 ± 0.4 g), underwent GTT (Figure 4H, dashed line), and were placed on HFD. After 2 weeks, mice weighed 24.5 ± 0.5 g. Repeat GTT showed marked deterioration (dotted line). The diet was then changed to HFD plus DFS. After 2 weeks, mice weighed 24.7 ± 0.4 g. GTTs were repeated and showed significant improvement (2.1 mmol/l in peak glucose), confirming an effect of DFS in another mouse line.

Increasing HIF-1α in human islets improved gene expression. Given the deleterious effects of β cell HIF-1α disruption and the beneficial effects of DFS in vivo, we examined the effects of increasing HIF-1α with DFO upon cultured human islets and compared this with the effects of hypoxia. Islets were cultured with DFO at the doses shown (μmol/l) or at normoxia (21% oxygen) or hypoxia (1%

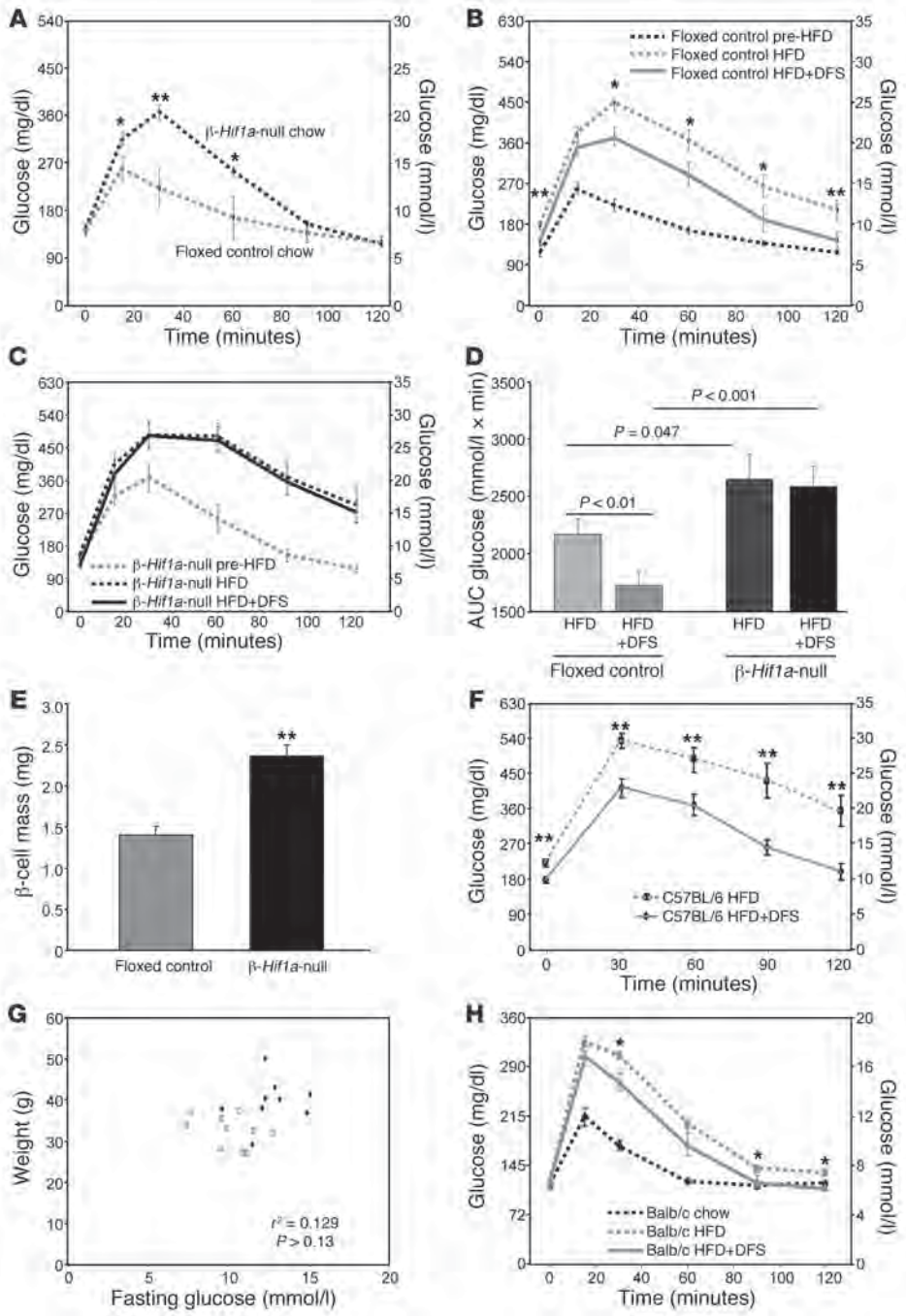


Figure 4
Lack of β cell HIF-1α leads to severe deterioration in glucose tolerance on a HFD and increasing HIF-1α levels with DFS improves glucose tolerance on a HFD. (**A**) β-*Hif1a*-null mice (n = 10) had worse glucose tolerance than floxed control littermates (n = 17). (**B**) On HFD, glucose tolerance deteriorated in floxed controls and improved following DFS. (**C**) On HFD, glucose tolerance deteriorated markedly in β-*Hif1a*-null mice, and there was no improvement with DFS. (**D**) Glucose tolerance AUC for mice at completion of the HFD and HFD plus DFS stages. (**E**) β cell mass was increased in β-*Hif1a*-null mice at study completion. (**F**) Glucose tolerance was significantly better in C57BL/6 mice receiving HFD plus DFS versus mice receiving HFD alone (n = 10 per group). (**G**) Weight and fasting glucose were not significantly correlated in the mice. Rectangles indicate mice receiving HFD plus DFS, and triangles indicate mice receiving HFD alone. (**H**) Balb/c mice had deterioration in glucose tolerance on HFD (dotted line) compared with chow (dashed line). Their glucose tolerance improved significantly on HFD plus DFS (n = 12). *P < 0.05 and **P < 0.01.

oxygen). The human therapeutic dose is approximately 125 μmol/l. DFO caused dose-dependent increases in *HNF4A*, *GLUT1*, *GLUT2*, *AKT2*, and *IRS2* mRNAs (Figure 5A). In contrast, hypoxia increased *GLUT1* but not *GLUT2*, *AKT2*, or the other genes shown (Figure 5B). Interestingly, there was a small but significant increase in *ARNT* mRNA with DFO (Figure 5C), which was not seen with hypoxia (Figure 5D). There was a significant increase in GSIS at moderate hyperglycemia in DFO-cultured human islets compared with control-cultured islets from the same donors (Figure 5E). The approximately 60% increase at high glucose was usual for recently isolated human islets. As expected, GSIS was not improved in hypoxic human islets and declined with longer exposure (Figure 5F).

Hif1a regulated Arnt. Having observed a small increase in *ARNT* expression with DFO treatment in normal human islets, we examined the effects of manipulating HIF-1α upon *Arnt* expression. RNAi-mediated knockdown of *Hif1a* in Min6 cells caused a more than 80% decrease in *Arnt* expression (Figure 6A; P < 0.01), and islets from β-*Hif1a*-null mice had a 50% decrease in *Arnt* expression (Figure 6B; P < 0.01), which was similar to the reduction in *Hif1a* itself. This was paralleled by a decrease in ARNT protein in immunostained pancreas of β-*Hif1a*-null versus floxed control mice (Figure 6C, compare bottom and top panels). Together these experiments show that decreasing HIF-1α in islets and β cells led to decreased *ARNT*.

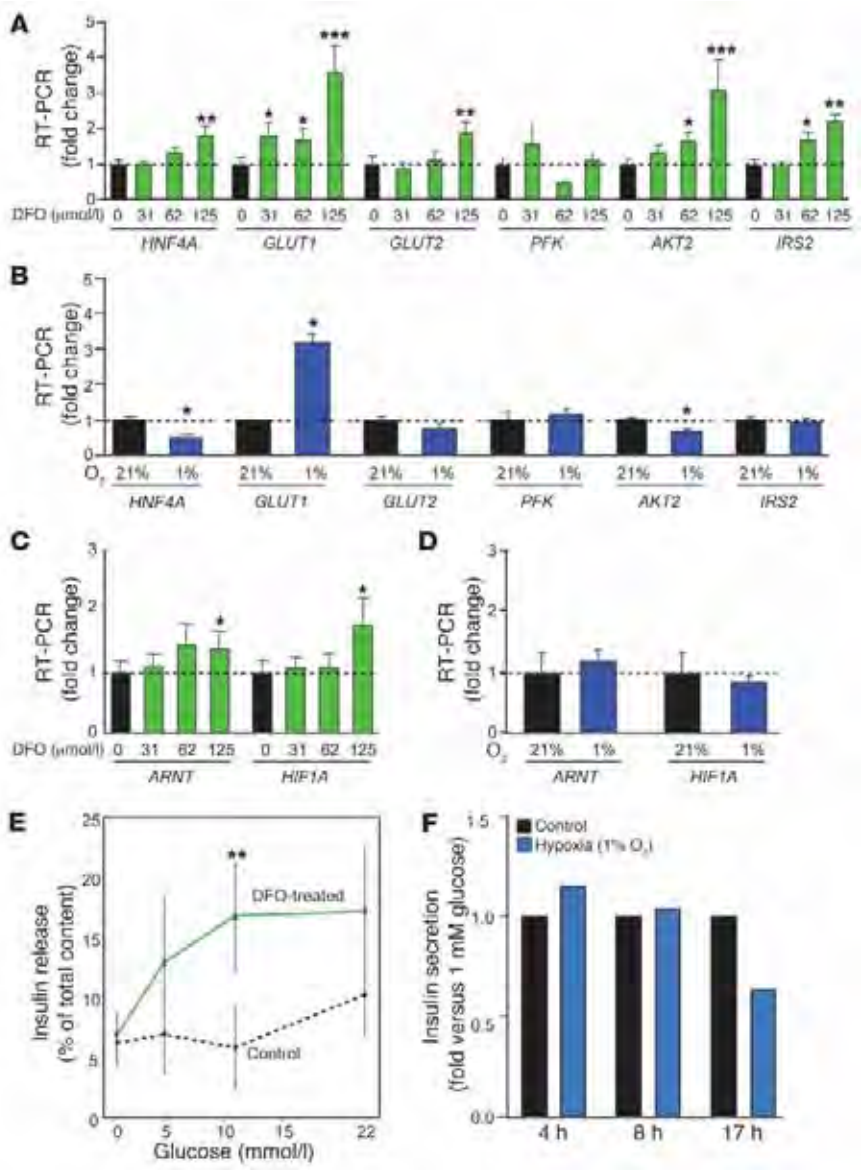


Figure 5
DFO improves gene expression and insulin secretion from human islets. (**A**) DFO increased expression of several genes in isolated human islets. (**B**) Hypoxic culture increased *GLUT1* expression but decreased that of *HNF4A* and *AKT2*. (**C**) DFO increased expression of *ARNT* and *HIF1A*. (**D**) Hypoxia did not alter *ARNT* or *HIF1A* expression. (**E**) DFO increased insulin secretion in isolated human islets. (**F**) GSIS was not improved in hypoxic islets and declined with longer exposure. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Increasing HIF-1α in human T2D islets increased ARNT, HNF4A, and G6PI expression. Human islets isolated from a new cohort of 3 people with T2D had significantly decreased *HIF1A* and *ARNT* expression (Figure 1A and Figure 6D). We examined the effect of culturing T2D islets with DFO. In this new group of T2D donors, we also found a more than 80% decrease in *ARNT* expression (Figure 6D). *HNF4A* and *G6PI* were also decreased, consistent with our previous report (Figure 6, E and F). Culture of islets from the same T2D donors with DFO increased *ARNT* to near-normal levels (Figure 6D; $P < 0.01$). DFO increased *HNF4A* and *G6PI* expression to near-normal levels (Figure 6, E and F; $P < 0.01$ for both genes). Similar results were also seen for *AKT2* (data not shown).

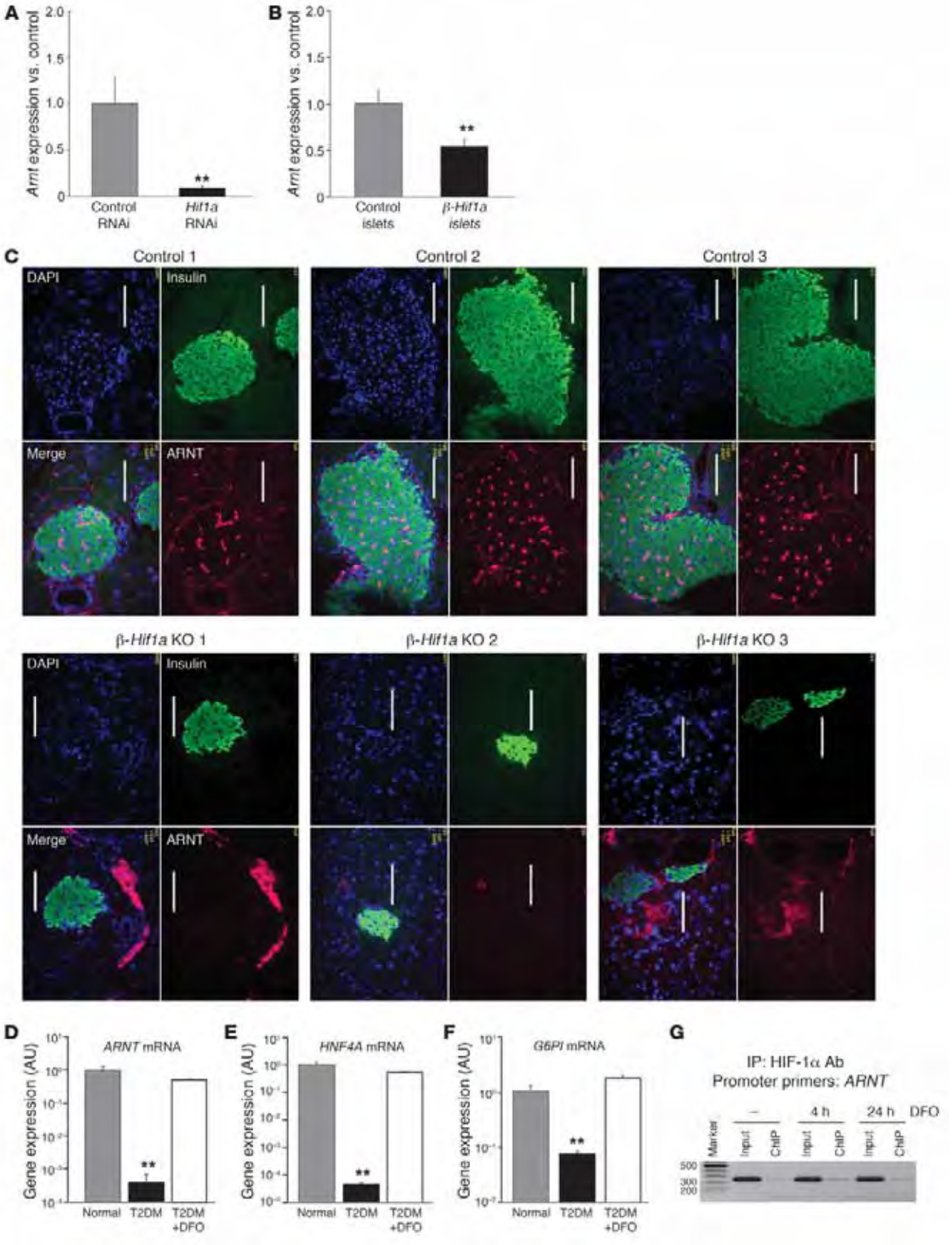
To determine whether HIF-1α bound directly to the *Arnt* promoter, we performed ChIP assays. In Min6 cells, HIF-1α antibodies pulled down the proximal *Arnt* promoter (Figure 6G). The amplified sequence (primers in Methods) contains a potential hypoxia-response element, GCGTG.

Differential effects with different methods of increasing HIF-1α. We examined the effects of hypoxia, *Vhl* knockdown, and DFO in isolated islets and Min6 cells with hypoxia in order to examine the mechanisms behind the different effects of DFO/DFS compared with hypoxia and to investigate the different results compared with those reported with *Vhl* knockouts (46–48). As shown in Figure 5F and Figure 7A, 1% oxygen did not promote GSIS. In human islets (Figure 7B) and Min6 cells (Figure 7C), 1% oxygen also did not increase expression of *GLUT2* or *AKT2*. As expected, 1% oxygen increased *Glut1* expression in Min6 cells. Interestingly, changes in gene expression differed between 5% oxygen and 1% oxygen. In cells exposed to 5% oxygen, *Glut1*, *Glut2*, and *Akt2* increased (Figure 7D). RNAi-induced *Vhl* knockdown in Min6 cells decreased *Vhl* mRNA by 33%. This led to a small but significant increase in *Glut2* (Figure 7E) and a nonsignificant increase in GSIS (Figure 7F). By doubling RNAi concentrations, approximately 5 5% *Vhl* knockdown was achieved. This led to decreased *Hnf4a* and *Akt2* expression, and the increase in *Glut2* was lost (Figure 7G). This was accompanied by a nonsignificant impairment in GSIS (Figure 7H).

Increasing HIF-1α levels by transfecting a proline-to-alanine mutant caused significant impairment in GSIS (Figure 7I). This was associated with the expected increases in *Hif1a* (>29-fold) and *Glut1* (>3-fold). However, there was also a significant decrease in expression of *Gck* (Figure 7J). Interestingly, there was significantly decreased total insulin content in the mutant-HIF-1α–transfected cells (approximately 50% of vector transfected; Figure 7K).

Removing iron by chelation improved HIF-1α activity and β cell function. The effect of adding iron in the form of ferric citrate was studied. At high doses, there was obvious cell death. At lower doses, there were significant decreases in *Hif1a*, *Hnf4a*, *Glut1*, and *Glut2* expression (Figure 7L). This identifies iron as a potential regulator of *HIF1A* in β cells. There was also significantly impaired GSIS, with only a nonsignificant 13% increase, following high glucose in iron-treated cells ($P = 0.017$ versus control high glucose).

DFO treatment at 16-times the therapeutic dose (2,000 μmol/l) also decreased *GLUT2* by 34% (Figure 8). Changes in *GLUT2* expression with various treatments are compiled in Figure 8. *Glut2* generally corresponded to GSIS, with lower *Glut2* expression and lower


A
Arnt expression vs. control
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β-Hif1a KO 3
DAPI
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HNF4A mRNA
Gene expression (AU)
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IP: HIF-1α Ab
Promoter primers: ARNT
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4 h
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Figure 6
HIF-1α regulates expression of *ARNT* and downstream genes. (**A**) *Hif1a* RNAi in Min6 cells decreased *Arnt* expression. (**B**) β-*Hif1a*-null mice had decreased *Arnt* mRNA compared with floxed controls. (**C**) ARNT protein was decreased in β-*Hif1a*-null islets versus floxed controls. Scale bar: 50 μm. (**D**) *ARNT* mRNA was decreased in islets from people with T2D. DFO increased *ARNT* expression to levels that did not differ significantly from normal. (**E**) *HNF4A* mRNA was decreased in islets from people with T2D and was increased by DFO. (**F**) *G6PI* expression was decreased in islets from people with T2D and was increased by DFO. (**G**) HIF-1α associated with the proximal *Arnt* promoter by ChIP. $^{**}P < 0.01$.

insulin secretion in β-*Hif1a*-null islets after HIF-1α knockdown with ferric citrate, reported for genetic VHL deletion (46, 47), and with toxic doses of DFO.

Discussion

People with T2D characteristically have pronounced impairment of first-phase insulin secretion (50–52). This defect is intrinsic, as it persists in isolated islets (53, 54) and is relatively glucose specific (54, 55) in the earlier stages of the disease. In previous studies, we demonstrated that T2D islets had decreased *ARNT* expression and, using gene inactivation approaches, showed that this could contribute to altered β cell function. In the present study, we show that T2D islets had decreased HIF-1α, which we show is also important for islet function. Deletion of HIF-1α in C57BL/6 mice resulted in impaired ATP generation and impaired glucose tolerance, accompanied by altered gene expression. Similar results were found in Min6 cells using RNAi. The β cell defect was relatively glucose specific, with only approximately 25% impairment in KCl-stimulated insulin release.

Although HIF-1α protein is tightly regulated, several methods of increasing it exist. These include hypoxia, decreasing VHL protein, mutation or decreased expression of the prolyl hydroxylases, treatment with heavy metals such as cobalt chloride, and iron chelation. Severe hypoxia and cobalt chloride are toxic. Genetic modification is not usually a therapeutic option for humans, although the future possibility exists with antisense RNA strategies. Thus, we studied the effects of iron chelation with DFS or DFO.

Treating mice made diabetic by high-fat feeding with DFS improved glucose tolerance. DFS was also effective in C57BL/6 and Balb/c wild-type mice but was completely ineffective in mice lacking β cell HIF-1α, demonstrating that HIF-1α is required for the benefit. Our HFD had 45% of calories from fat, compared with 12% in normal chow. While this is high, the average fat intake in the American diet is more than 30%. The top 20% of the population consume 46% of calories from fat (56, 57). DFS was effective despite continuing the HFD, suggesting that it may be effective in people with T2D, in whom high fat-intake is common.

Surprisingly, DFO treatment normalized *ARNT* and other genes in T2D islets. HIF-1α is predominantly regulated at the protein level, and DFO treatment was apparently sufficient to normalize HIF-1α function, as assessed by expression of downstream genes. The magnitude of effect on *ARNT*, *HNF4A*, and *G6PI* was large in T2D islets (>10-fold), in which basal HIF-1α was low. This is the first time that a strong regulator of *ARNT* expression has been identified. In contrast, the change in *ARNT* expression in normal islets, in which HIF-1α was not low at baseline was modest. *Hif1a* itself was decreased by treatment with iron.

In other cell types, HIF-1α regulates *PDK1* (10, 11), *COX4.1*, *COX4.2*, and LON protease changes associated with increased ATP (58). Consistent with these studies, we observed that decreasing HIF-1α decreased ATP. ATP generation is required in β cells for sensing of glucose, which in turn triggers insulin release. Furthermore, impaired ATP generation in our models was associated with impaired insulin release, consistent with HIF-1α being a regulator of β cell energy homeostasis and insulin release. Thus, decreased HIF-1α impaired glucose-stimulated ATP generation, providing the mechanism by which decreased availability of a transcription factor can cause β cell dysfunction.

Recently, 3 groups reported clear, adverse effects of homozygous deletion of VHL upon β cell function (46–48). In these models, there was a massive increase in HIF-1α protein. Disruption of *VHL* was accompanied by adverse gene expression changes, increased lactate, and severely impaired GSIS. These findings are interesting but were unexpected as heterozygous whole-body VHL knockout mice appeared grossly normal (59), with 5%–25% of mice developing abnormal vascular lesions in later life on some genetic backgrounds (60). People with VHL syndrome may develop endocrine pancreatic tumors, pancreatic cysts, and occasional insulinomas (16, 61–63). However, less than 3% of patients are reported to have abnormal glucose tolerance, despite frequently requiring steroids and/or pancreatic surgery (16, 61, 62, 64). This suggests that a heterozygous germline mutation, as occurs in VHL patients, may not cause an increased risk of diabetes. Mutations in subunits of the succinate dehydrogenase complex (65, 66) and the HIF-1α prolyl hydroxylases are also associated with increased HIF-1α protein, but there are no reported alterations in diabetes incidence.

Loss of VHL was associated with decreased *Glut2* mRNA (46, 47). We also found decreased *Glut2* with DFO at 16-times the therapeutic dose, exposure to 1% oxygen, high-dose VHL RNAi, and supplemental iron. In contrast, therapeutic levels of DFO and 5% oxygen treatment both caused different changes in gene expression, and in particular, *Glut2* was increased. The different changes in gene expression with different methods of increasing HIF-1α were in accordance with the changes in β cell function (Figure 8).

Thus, there appears to be a dose-response curve for HIF-1α (Figure 8). Deletion is deleterious in C57BL/6 mice and Min6 cells. Mild increases are beneficial for β cell function and glucose tolerance but very high levels, such as those achieved with homozygous VHL deletion, severe hypoxia, or overexpression of a degradation resistant mutant, are clearly deleterious for β cell function.

Hydroxylation and proteolysis of HIF-1α requires iron, which is chelated by DFO and DFS. Iron overload due to transfusion dependency or hemochromatosis can cause β cell dysfunction and increases diabetes incidence (67, 68). It is perhaps less widely recognized that in the absence of transfusion-dependent iron overload or hemochromatosis, increases in serum ferritin or transferrin saturation are associated with increased risk of diabetes and the metabolic syndrome (69–74). High dietary iron intake is also associated with diabetes (71, 75). Conversely, venesection and blood donation can improve β cell function in people with diabetes (70, 76). Regular blood donation has been reported to protect against diabetes, as does a vegetarian diet (67, 70, 76). Disruption of the HIF-1α–partner ARNT in endothelial cells leads to pronounced iron accumulation in the liver (77), suggesting the intriguing potential for a vicious cycle of decreased HIF-1α, decreased ARNT, increased iron accumulation, and decreased HIF-1α. Based on our data and the absence of a DFS effect in mice lacking β cell HIF-1α,

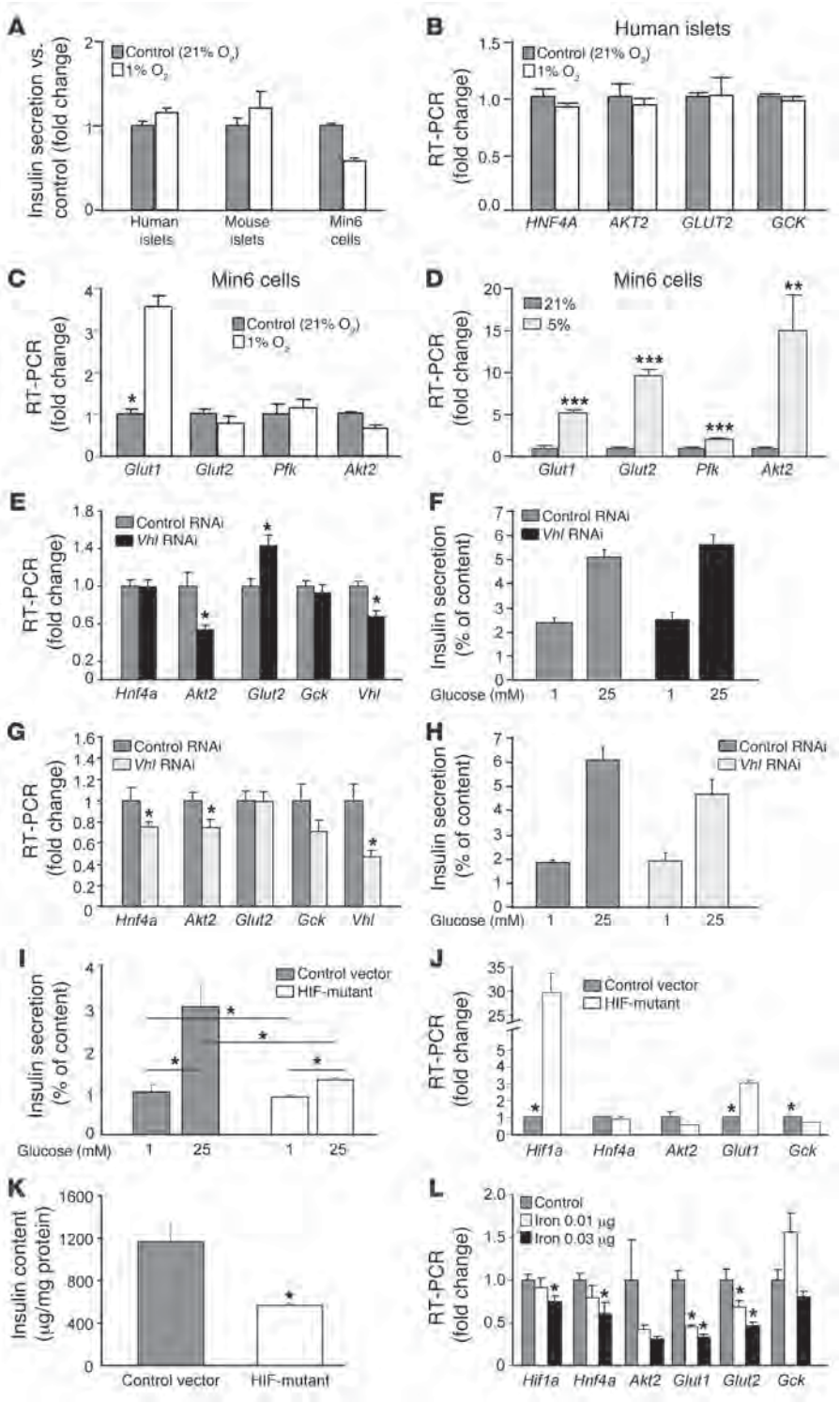

Figure 7
The differing effects on gene expression and insulin secretion with increasing HIF-1α levels using hypoxia or *VHL* RNAi. (**A**) Insulin secretion was not improved in human islets, mouse islets, or Min6 cells cultured under hypoxic conditions. (**B**) One percent oxygen did not increase *GLUT2* expression in human islets. (**C**) One percent oxygen did not increase *Glut2* expression in Min6 cells. (**D**) Moderate hypoxia (5% oxygen) increased *Glut2* expression. (**E**) Modest *Vhl* knockdown (35%) increased expression of *Glut2* and was associated with a nonsignificant increase in insulin release (**F**). (**G**) High-dose *Vhl* knockdown achieved a 55% decrease in *Vhl* and did not increase *Glut2* expression. (**H**) High-dose *Vhl* RNAi lowered insulin secretion nonsignificantly. (**I**) Transfection with proline-to-alanine mutant HIF-1α significantly impaired insulin secretion. (**J**) *Hif1a* expression was increased more than 29-fold and was accompanied by increased *Glut1* expression and decreased *Gck*. (**K**) Total insulin content was decreased in the proline mutant HIF–overexpressing cells. (**L**) Ferric citrate treatment significantly decreased *Hif1a* expression and was accompanied by decreased expression of *Hnf4a*, *Akt2*, *Glut1*, and *Glut2*. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

we postulate that a decrease in HIF-1α may be a mechanism contributing to the increased risk of diabetes with increased iron.

In summary, these studies demonstrate that β cell HIF-1α is important for β cell reserve, and increasing HIF-1α by iron chelation markedly improved glucose tolerance on a HFD. Increasing HIF-1α normalized gene expression in T2D islets. Therefore, we propose that increasing HIF-1α by iron chelation may be a valid therapeutic strategy for the treatment of human T2D.

Methods

Human islet studies were approved by St. Vincent's Clinical School Human Research Ethics Committee. All participants gave informed consent. Animal studies were approved by the Garvan Institute Animal Ethics Committee. Human islets were purified using the modified Ricordi method as previously described (22). RNA was isolated using Qiagen RNeasy kits. Gene expression was measured by real-time PCR using the Invitrogen RT-for-PCR kit. The second step was performed in an ABI Prism 7700 Sequence Detection

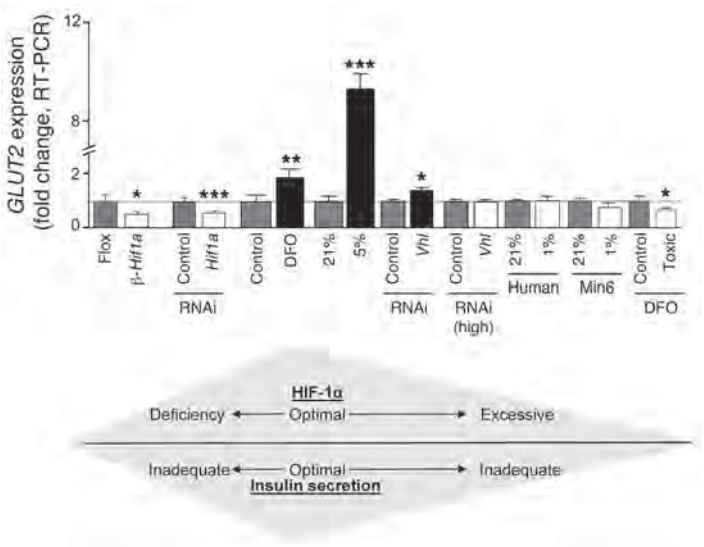

Figure 8
Modest increases in HIF-1α improve insulin secretion. Changes in HIF-1α, which were associated with decreased *GLUT2*, were associated with impaired insulin secretion. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

System (Applied Biosciences) with LightCycler-RNA Master SYBR Green I (Roche). Primers are in Supplemental Table 1. Every plate included a control gene (TATA-box binding protein/*TBP*) for every subject.

Immunohistochemistry and antibodies. Slides were cut from paraffin-embedded pancreata. Antibodies were purchased from Novus Biologicals (HIF-1α, HIF2α/EPAS1), Orbigen (AhR), Cell Signaling Technology (insulin), or BD Biosciences (ARNT). Primary antibodies were applied overnight at 4°C. Secondary antibodies were Cy2, Cy3, or Cy5 conjugated and applied for 1-hour at room temperature. Slides were viewed on a Zeiss inverted microscope and images were taken with AxioVision software. For each figure, the images were taken in the same session with identical camera settings. For each antibody, species-matched nonimmune immunoglobulin and secondary antibody alone were tested as negative controls. HIF-1α antibody specificity was additionally supported by the lack of β cell HIF-1α staining in knockout mice.

Generation of β-Hif1a-null mice. β-*Hif1a*-null mice were generated using the Cre-lox system. Mice, with floxed HIF-1α (49), were bred with mice expressing Cre-recombinase, under control of the rat insulin promoter (RIP-Cre mice). In our colony, RIP-Cre mice did not have abnormal glucose tolerance (Figure 2A). Recombination efficiency was estimated by semiquantitative PCR, using the genotyping primers (49) at 60%–80%. Anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology Inc. ARNT affinity purification was done by binding ARNT antibody (12 μg) to 1 ml of packed protein A/G beads in 5 ml columns. Unbound antibody was removed by washing with 20 ml of PBST. Min6 cells (a gift from J. Miyazaki, Physiological Chemistry, Osaka University, Osaka, Japan; ref. 78) were grown to 80%–90% confluence, washed twice in PBS, and placed in serum-free high glucose DMEM for 4 hours with or without DFO. Cells were scraped into LID lysis buffer with protease inhibitors as previously described (79). Cytoplasmic extracts were collected after centrifugation. The nuclear-containing pellet was disrupted by sonication. Extracts were applied to columns, and the flow-through was reapplied twice to obtain maximal binding. After this, the columns were washed twice with 20 ml of LID buffer, followed by 2 washes with 20 ml of PBST. Bound proteins were eluted with reducing sample buffer and were size separated by 10% SDS-PAGE, followed by protein staining with Coomassie blue (Figure 1D).

For mass spectrometry, gel slices were digested with 5 ng/ml sequencing grade-modified trypsin (Promega) in 25 mM ammonium bicarbonate containing 0.01% n-octylglucoside for 18 hours at 37°C. Peptides were eluted from the gel slices with 80% acetonitrile and 1% formic acid. Tryptic digests were separated by capillary HPLC (C18, 75 mM i.d.; Picofrit column, New Objective), using a flow rate of 100 nl/min over a 3-hour reverse phase gradient, and analyzed using a LTQ linear Ion Trap LC/MS^{n} system (Thermo Electron). Resultant MS/MS spectra were searched against the NCBI Refseq database (http://www.ncbi.nlm.nih.gov/refseq/) (TurboSequest, BioWorks 3.1, Thermo Electron), with cross-correlation scores of greater than 1.5, 2.0, and 2.5 for charge states U′, u′, and Ʊ, respectively, more than 30% fragment ions, and a ranking of primary score (RsP) value of <3. Proteins were identified with more than 2 unique peptide matches.

Coimmunoprecipitation studies were performed using 2 μg of the indicated antibody and protein A/G beads and by incubating overnight with the indicated cell lysate, followed by washing, elution with reducing sample buffer, and separation by 10% SDS-PAGE. Proteins were detected with the indicated antibody, followed by the appropriate HRP-conjugated secondary antibody, and detection by enhanced chemiluminescence. For each antibody, species-matched nonimmune immunoglobulin and antibody-alone lanes were tested as negative controls to confirm antibody specificity.

Islet isolation from mice. Islets were isolated from mice as previously described (22). All mice except for the Balb/c mice were inbred C57BL/6 for at least 12 generations.

DFO and hypoxia treatment. DFO treatment was at 125 μM for 4 hours, unless otherwise specified. Hypoxia treatments were for 2 hours, unless otherwise indicated. Hypoxia was achieved with a hypoxic chamber and an oxygen sensor to confirm levels.

Alanine HIF-1α mutant. Proline residues 402 and 577, in the murine HIF-1α cDNA, were mutated by site-directed mutagenesis to alanine and the construct was cloned into the pcDNA3 vector and sequenced. The construct and the vector were transfected into Min6 cells using Lipofectamine 2000, according to the manufacturer's instructions, and selected using geneticin for 1 week. Total insulin content was measured and corrected for total protein, which was measured by DC Bradford assay.

In vivo testing. GTTs, GSIS, in vitro GSIS, β cell mass, and mRNA expression in islets were assessed as previously described (22). AUC for the GTTs was calculated using the trapezoidal method. Insulin tolerance tests were performed by injecting insulin at 0.5 U/kg and measuring glucose at the times shown.

Measurement of intracellular ATP concentrations. ATP concentrations were measured in islets and in Min6 cells following basal culture in 1 mM glucose for 1 hour, followed by washing and exposure to 1 mM or 25 mM glucose for 15 minutes. Cells were then placed on ice, washed twice in ice-cold PBS, and lysed. ATP was measured using the Roche Bioluminescence kit. Results were corrected for total protein.

RNAi treatment of Min6 cells and insulin release. Using Min6 cells, HIF-1α, HIF-2α, AhR, and ARNT were decreased by treatment with smartpool RNAi (Dharmacon) and transfected using Lipofectamine 2000 (Invitrogen), according to the respective manufacturers' protocols. Scrambled-sequence RNAi was used as a control in all experiments. Total RNAi concentrations were the same for the combination experiments (e.g., 3-times control versus HIF-1α plus AhR plus HIF-2α versus 2-times control plus HIF-1α). Cy3-labelled RNAi and FACS sorting were used to determine transfection efficiency, which was more than 75% (data not shown). Experiments were performed 48 hours after transfection. GSIS was assessed in triplicate wells in 3 separate experiments and corrected for total insulin content. In separate experiments, RNA was isolated for real-time PCR.

HFD studies. Male floxed control (n = 17) or β-*Hif1a*-null mice (n = 10) had GTTs as described above. They were then placed on a HFD, based on Rodent Diet no. D12451 from Research Diets Incorporated, which contained 45% of calories from fat (lard). DFS was thoroughly mixed into the vitamin mix during diet formulation to achieve a 30 mg/kg/d dose. This was calculated by measuring food intake of separate C57BL/6 mice on HFD (HFD [g]/mouse weight [g]/d) and calculating accordingly.

T2D culture with DFO. Islets were freshly isolated from 3 individuals with T2D. Islets were cultured overnight, in either control medium or control medium with 125 μM DFO, prior to RNA isolation.

ChIP. ChIP was performed using the Active Motif kit (Carlsbad), according to the manufacturer's instructions. The ARNT promoter primers were GCTTCCTAGCTCAGGCTTCC and AAGAGCCACTCCGCAGATTA, which produce a 250-bp band, which incorporates a GCGTG sequence.

Statistics. Statistics were calculated in Excel or in SPSS version 14. Unless otherwise specified, Student's t test with unequal variance was used to compare groups. For all figures, error bars indicate ± SEM. P values of less than 0.05 were considered significant.

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