

Analysis of Mouse Models of Insulin Secretion Disorders

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ABSTRACT

In this thesis, three mouse models and cell culture techniques were used to investigate genetic factors leading to glucose intolerance, diabetes or hyperinsulinemia. (1) Loss of nicotinamide nucleotide transhydrogenase (Nnt) function is linked to ROS-mediated uncoupling of mitochondrial metabolism and reduced insulin secretion. The enzymatic activity of Nnt generates mitochondrial NADPH essential for ROS detoxification. However, the exact nature of ROS as well as the antioxidant enzymes involved are still unknown. It was found that MIN6 cells in which Nnt was silenced displayed an increase in mitochondrial H_2O_2 upon stimulation with both the ROS generator menadione and glucose. Knockdown of GPx1, however, had no effect on mitochondrial H_2O_2 and was linked to a Ca^{2+} independent hypersecretion of insulin. Exogenous GSH did not increase the glucose-stimulated rise in $[\text{Ca}^{2+}]_i$ in Nnt mutant and control islets. This finding substantiates the suggested role of ROS as a signalling molecule in insulin secretion. In contrary to previous studies on MIN6 cells and single β -cells, the glucose-stimulated increase in $[\text{Ca}^{2+}]_i$, measured in intact Nnt mutant islets showed no difference compared with control islets. This might indicate differences in single β -cell versus whole islet physiology or be attributable to differences in genetic background.

(2) The activating V59M mutation in the K_{ATP} channel subunit Kir6.2 causes neonatal diabetes in humans. Transgenic mice constitutively expressing the V59M mutation in their β -cells recapitulated features of the disease phenotype in humans. In vitro studies showed that β -cells exhibited impaired first- and second-phase glucose-dependent insulin secretion. This was paralleled by a complete loss of the initial glucose-dependent rise in $[\text{Ca}^{2+}]_i$ in V59M islets. However, islets from mice carrying an uninduced Kir6.2-V59M mutation, or mice expressing Cre recombinase, showed no impairment of their $[\text{Ca}^{2+}]_i$ responses. If expression of the transgene was induced but mice were then treated with the K_{ATP} channel blocker glibenclamide to control their diabetes, isolated islets displayed a loss of the initial rise in $[\text{Ca}^{2+}]_i$ and a reduced sustained increase of $[\text{Ca}^{2+}]_i$, which was associated with abrogation of synchronous Ca^{2+} oscillations in β -cells. In the presence of glibenclamide, both basal Ca^{2+} levels and insulin secretion from isolated islets were elevated, whereas the glucose-stimulated $[\text{Ca}^{2+}]_i$ response, synchronicity of Ca^{2+} oscillations and insulin secretion were restored. Furthermore, Ca^{2+} imaging revealed that the number of β -cells per islet responding to glucose was similar to control islets, underlining the importance of early treatment with glibenclamide to achieve glycemic control.

(3) The E1506K mutation of the SUR1 subunit of the K_{ATP} channel causes a reduction in channel activity. It is associated with hyperinsulinism of infancy in early life and leads to the development of glucose intolerance, insulin deficiency and diabetes later in life. Islets isolated from a knock-in mouse expressing the SUR1-E1506K mutation showed enhanced glucose-stimulated insulin secretion from young het-E1506K (first- and second-phase) and young hom-E1506K islets (basal and first- and second-phase). Old hom-E1506K islets exhibited normal basal insulin secretion but first- and second-phase secretion was found to be markedly reduced in comparison to wild-type islets. This was due to a decline in insulin content. Confocal Ca^{2+} imaging suggested that this was not caused by β -cell loss. Measurements of $[\text{Ca}^{2+}]_i$ in young het-E1506K islets showed spontaneous Ca^{2+} oscillations in 2 mM glucose, which did not result in an average elevation in $[\text{Ca}^{2+}]_i$. However, young hom-E1506K islets displayed vigorous Ca^{2+} oscillations in 2 mM glucose that led to an average elevation in $[\text{Ca}^{2+}]_i$. Depolarisation of E1506K islets with either glucose, tolbutamide or KCl produced a reduced increase in $[\text{Ca}^{2+}]_i$ compared to wild-type islets indicating either a reduction in Ca^{2+} influx or an enhanced clearance of Ca^{2+} . Peak voltage-dependent inward currents recorded from β -cells in het-E1506K islets were larger than in wild-type β -cells. Untypically for mouse β -cells, these inward currents contained a Na^+ current resulting from a shift in its inactivation towards a more positive membrane potential.

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DECLARATION

The work in this thesis is my own, except for the following experiments:

Blood glucose levels of transgenic mice following induction of the V59M transgene (Fig. 4.5), insulin secretion from perfused control and ib-V59M islets (Fig. 4.11) and Insulin secretion and insulin content from perfused wild-type and E1506K islets (Fig. 5.2) were performed by Dr Kenju Shimomura. Electrophysiological studies on E1506K islets (Fig. Y.7-8) were carried out together with Dr Quan Zhang.

ABBREVIATIONS

ADP	adenosine diphosphate	GPx	glutathione peroxidase
AGE	advanced glycation end products	GR	glutathione reductase
ANOVA	analysis of variance	GSH	glutathione (reduced form)
ATP	adenosine triphosphate	GSSG	glutathione (oxidised form)
avg	average	GTP	guanosine triphosphate
B2M	beta-2 microglobulin	HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid
bp	base pair		
BSA	bovine serum albumin	het	heterozygous
cAMP	cyclic adenosine monophosphate	HI	hyperinsulinism of infancy
cDNA	complementary DNA	hom	homozygous
cGMP	cyclic guanosine monophosphate	HRP	horseradish peroxidase
cm	centimetre	JNK	c-Jun N-terminal kinase
DAG	diacylglycerol	K _{ATP} channels	ATP-sensitive potassium channels
DNA	deoxyribonucleic acid	K _{Ca}	Ca ²⁺ -activated potassium channels
e ⁻	electron	kDa	kilodalton
E1506K	glutamic acid to lysine mutation at codon 1506	Kir	inwardly rectifying potassium channels
EC	extracellular	KRB	Krebs Biocarbonate Buffer
EGTA	ethylene glycol tetraacetic acid	Kv	voltage-gated potassium channels
ELISA	enzyme-linked immunosorbent assay	l	litre
Epac2	cAMP-guanosine nucleotide exchange factor 2	log	common logarithm
F	fluorescence intensity	M	molar
F6P	fructose 6-phosphate	MafA	musculoaponeurotic fibrosarcoma oncogene family, protein A
FADH ₂	flavin adenine dinucleotide (reduced form)	MAPK	mitogen-activated protein kinases
FBP	fructose 1,6-bisphosphate	mg	milligramme
FBS	foetal bovine serum	MgADP	magnesium ADP
g	gravitational force (9.8 metres per second squared)	MgATP	magnesium ATP
g	gramme	min	minutes
G	glucose	ml	millilitre
G6P	glucose 6-phosphate	mM	millimolar
G745D	glycine to aspartic acid mutation at codon 745	ms	millisecond
Gapdh	glyceraldehyde 3-phosphate dehydrogenase	mV	millivolt
GK	glucokinase	n	number
GLUT	glucose transporter	N68K	asparagine to lysine mutation at codon 68
		NADH	nicotinamide adenine dinucleotide (reduced form)

NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)	TTX	tetrodotoxin
NBD	nucleotide-binding domain	TXNIP	thioredoxin-interacting protein
NCX	Na ⁺ /Ca ²⁺ exchanger	UCP2	uncoupling protein 2
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells	UV	ultraviolet
ng	nanogramme	V59M	valine to methionine mutation at codon 59
nM	nanomolar	VDCC	voltage-dependent Ca ²⁺ channels
nm	nanometre	ΔΨ	membrane potential
Nnt	nicotinamide nucleotide transhydrogenase	°C	degree Celsius
NOX	NADPH oxidase	μg	microgramme
ns	nonsense	μl	microlitre
pA	picoampere	μM	micromolar
PCR	polymerase chain reaction		
Pdx-1	pancreatic and duodenal homeobox 1		
pF	picofarad		
PFK-M	Phosphofructokinase (M-type isoform)		
PKA	protein kinase A		
PKC	protein kinase C		
PLC	phospholipase C		
PMCA	plasma membrane Ca ²⁺ -ATPase		
POMC	pro-opiomelanocortin		
Prx	peroxiredoxin		
RNA	ribonucleic acid		
ROS	reactive oxygen species		
rpm	rounds per minute		
RT	room temperature		
SAPK	stress-activated protein kinase		
sec	seconds		
SEM	standard error of the mean		
SERCA	sarco-endoplasmic reticulum Ca ²⁺ -ATPase		
shRNA	short hairpin RNA		
siRNA	short interfering RNA		
SOD	superoxide dismutase		
SUR	sulphonylurea receptor		
T2DM	type 2 diabetes mellitus		
TCA	tricarboxylic acid		
TEA	tetraethylammonium		
TMD	transmembrane domain		
tol	tolbutamide		
tris	tris(hydroxymethyl)aminomet hane		

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1 GENERAL INTRODUCTION

1.1 REGULATION OF BLOOD GLUCOSE AND THE ISLET OF LANGERHANS

All cells in our body rely on the oxidative degradation of complex nutrient molecules such as carbohydrates, lipids or proteins to generate energy for driving vital physiological functions or synthesising biological molecules. These exergonic reactions often require the presence of oxygen to enable a complete breakdown of nutrients (mostly glucose) inside the cell. However, the intake of food is intermittent, which leads to the risk of depletion of reactants. This necessitates a system of metabolic regulation that enables excess fuel to be absorbed, stored and released for use during periods when no food is ingested.

The key hormonal regulators of glucose metabolism are glucagon and insulin. Insulin and glucagon are peptide hormones secreted by, respectively, the β -cells and α -cells of the pancreatic islets of Langerhans. Each islet is an oval, hundred micrometer-long mini organ that is highly vascularised and contains endocrine cells, endothelial cells, nerves and fibroblasts (Brissova and Powers 2008). In mice, pancreatic islets are composed of about 75% β -cells, 20% α -cells and 5% δ -cells that produce somatostatin (Cabrera, Berman et al. 2006). Additionally, they contain a small percentage of polypeptide-producing PP-cells (Larsson, Sundler et al. 1976).

Whereas release of somatostatin and most importantly glucagon into the blood stream elevates blood glucose levels, insulin is the only glucose-lowering hormone. Insulin exerts its hypoglycemic effects by binding to the insulin receptor on target cells, mainly muscle, liver and fat cells, and thereby enhancing glucose transport into these cells.

Once the body's energy needs are met, glucose is converted to glycogen or fat (particularly in adipose tissue). The major target of glucagon is the liver where it: (1) promotes the breakdown of glycogen to glucose (glycogenolysis) and the subsequent release of glucose into the bloodstream; and (2) stimulates the synthesis of glucose from lactic acid, amino acids and lipids (gluconeogenesis). Hence, insulin and glucagon work in concert to maintain the blood glucose concentration at a stable level (glucose homeostasis). See figure 1.1.

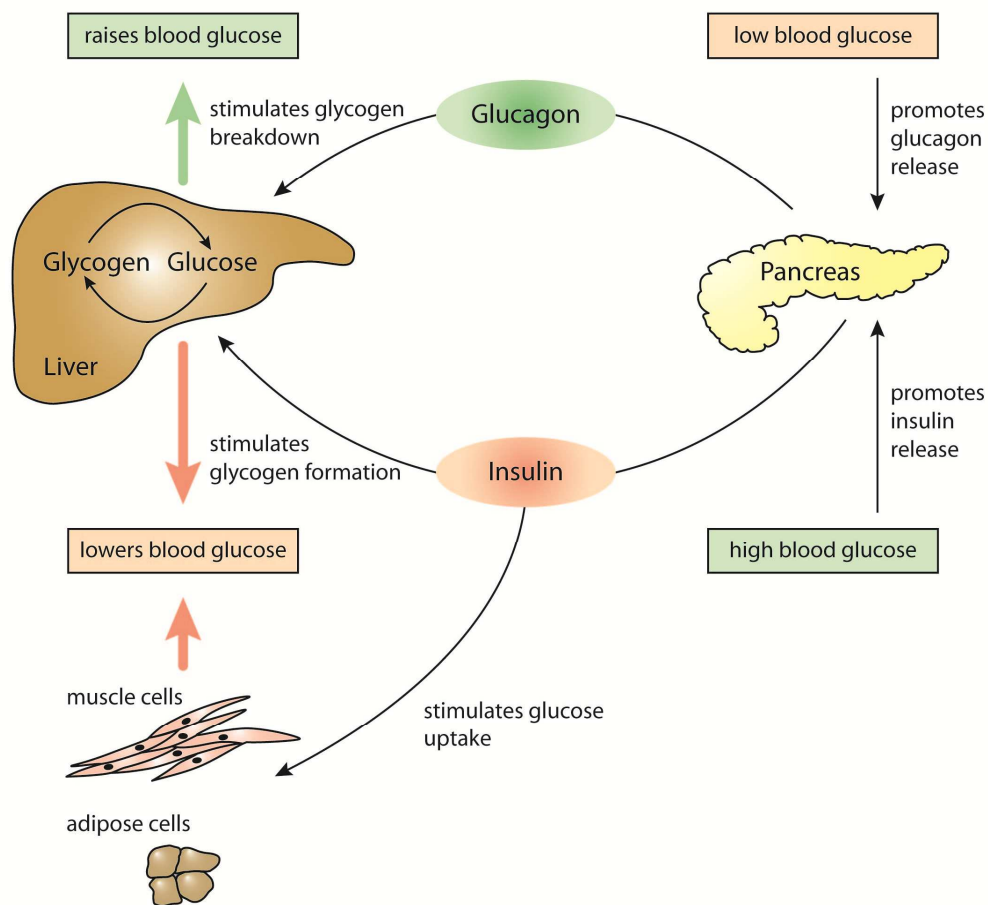


Fig. 1.1 Maintenance of glucose homeostasis

In response to high blood glucose levels, insulin is released from the pancreas and stimulates glucose uptake by muscle, liver and fat cells, resulting in a decrease of blood glucose. Glucagon, released in response to a fall in blood glucose, triggers glycogen breakdown and thereby raises the blood glucose level.

1.2 HYPOGLYCEMIA AND DIABETES MELLITUS

A permanent or prolonged disturbance of glucose homeostasis can either lead to hypoglycemia or to diabetes mellitus. Hypoglycemia can be caused by excess unregulated insulin secretion, which leads to enhanced glucose uptake into target tissues and reduced release of glucose into the blood. This is because insulin has an inhibitory effect on glycogenolysis and gluconeogenesis. Insulin also suppresses the stimulatory effect of glucagon on ketogenesis and lipolysis, thereby reducing alternative energy substrates. Thus, unregulated insulin secretion deprives the brain of its primary (glucose) and secondary (ketone bodies) energy sources and, when untreated, can lead to hypoglycemic brain injury and mental retardation (Hussain, Blankenstein et al. 2007).

Diabetes mellitus is characterised by hyperglycemia and is conventionally divided into two major forms. Type 1 diabetes is an autoimmune disease in which the patient's own immune system reacts to islet antigens and destroys the β -cells. Type 2 diabetes mellitus (T2DM) is a heterogeneous polygenic disorder with an important environmental component. Factors such as obesity, insufficient physical activity, and excessive carbohydrate intake enhance the risk of genetically predisposed people becoming diabetic (Robertson 2006). T2DM is determined by two distinct abnormalities: abnormal β -cell function and decreased insulin sensitivity (Bell and Polonsky 2001). Both β -cell dysfunction and insulin resistance are closely intertwined, and together cause further deterioration of insulin secretion and increase insulin resistance. In an attempt to maintain glucose homeostasis in the face of hyperglycemia and insulin resistance, the pancreatic β -cell adapts by secreting more insulin. This stress gradually causes β -cell deterioration and accelerated apoptosis (Butler, Janson et al. 2003; Aston-Mourney, Wong et al. 2007). It is now recognized, however, that abnormalities in insulin secretion precede the onset of T2DM and that the disease can occur in the absence of insulin resistance (Hosker, Rudenski et al. 1989; Del Prato and Tiengo 2001; Del Prato 2003). Finally, there are also monogenic forms of diabetes: they are discussed more fully later.

1.3 INSULIN SECRETION

Although the control of insulin secretion is complex and combines inputs from metabolic stimuli such as amino acids or sugars, hormones and the autonomic nervous system, glucose emerges as the most important regulator of insulin secretion (Henquin, Ravier et al. 2003; Ashcroft 2005). Pancreatic β -cells act as elaborate glucose sensors that adjust the secretion of insulin according to the blood glucose level (Fig. 1.2). Fluctuations in glucose plasma levels lead to glucose equilibration across the plasma membrane of the β -cell, mediated by the GLUT2 glucose transporter (Johnson, Newgard et al. 1990), which has a low affinity for glucose. After glucose has entered the cell, it is phosphorylated to glucose-6-phosphate by glucokinase (GK), which is also characterised by a low affinity for its substrate. This reaction traps glucose inside the cell and determines the flux rate for glycolysis (Matschinsky 1996). Metabolism of glucose by glycolysis, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation produces ATP. The increase of ATP and fall of MgADP close ATP-sensitive potassium channels (K_{ATP} channels), which set the resting conductance of the β -cell, leading to a depolarization of the β -cell (Ashcroft, Harrison et al. 1984). Membrane depolarization stimulates the influx of Ca^{2+} through plasma membrane voltage-gated Ca^{2+} channels (mainly L-type channels) resulting in the exocytosis of insulin granules (Keahey, Rajan et al. 1989; Ammala, Eliasson et al. 1993; MacDonald, Joseph et al. 2005).

In addition to this triggering pathway, the β -cell is endowed with a metabolic amplifying pathway (Henquin 2000; Henquin 2009; Henquin, Nenquin et al. 2009). The amplifying pathway is only operational once $[Ca^{2+}]_i$ has been elevated (by the triggering pathway) and augments the action of Ca^{2+} on exocytosis. The effect of glucose metabolism on the amplifying pathway can be observed in isolation from that on the triggering pathway when β -cell $[Ca^{2+}]_i$ is elevated by depolarisation with either a K_{ATP} channel blocker or by a depolarising concentration of K^+ in the presence of K_{ATP} channel opener. Such studies show that the amplifying pathway is independent of whether the K_{ATP} channel is open or closed.

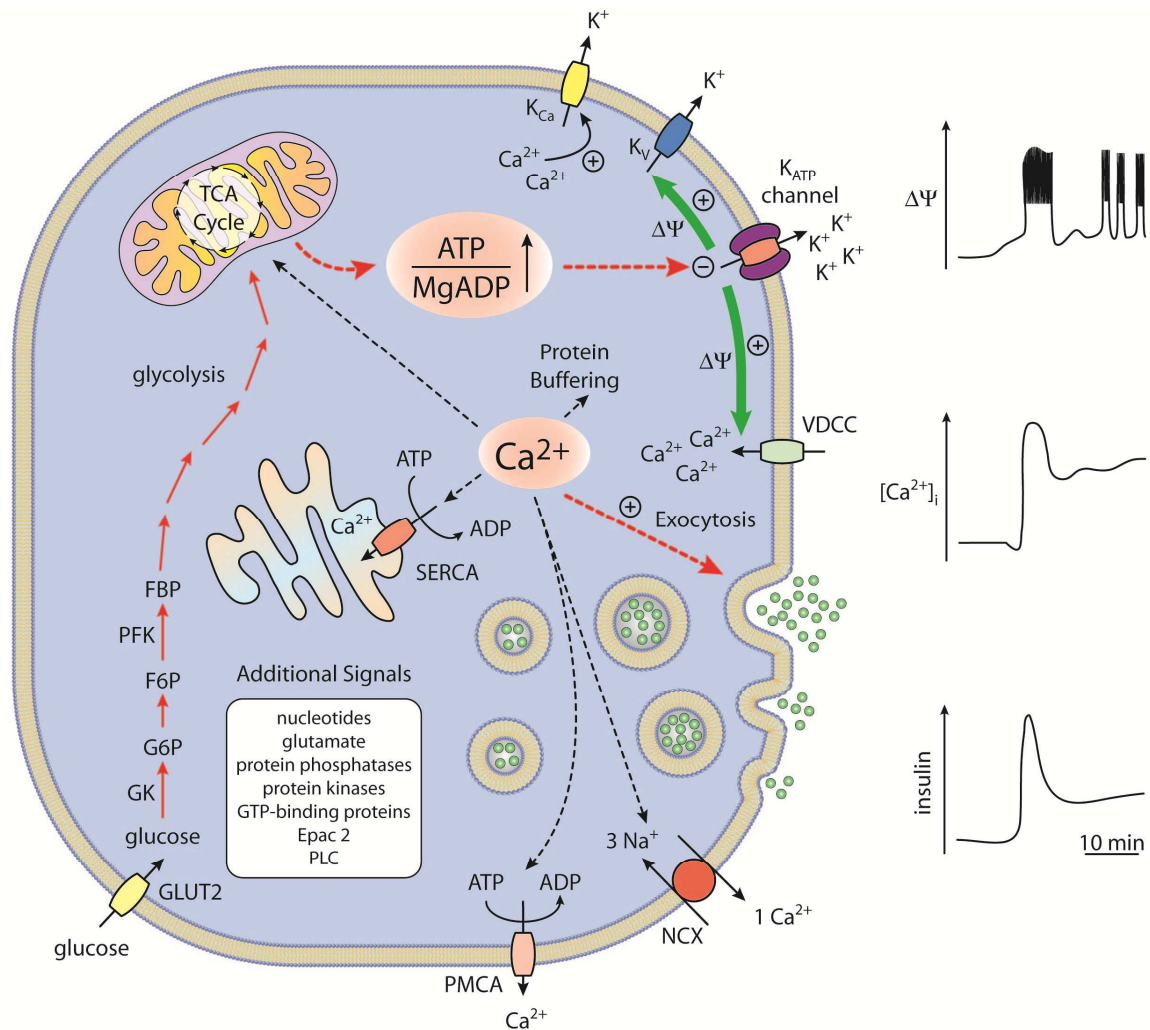


Fig. 1.2 Glucose-stimulated insulin secretion by the β -cell

When blood glucose levels rise, e.g. after a meal, glucose is taken up into the β -cell by glucose transporters (e.g. GLUT2) and oxidized via glycolysis, the TCA cycle and oxidative phosphorylation leading to an increase in ATP and a fall in MgADP. Upon binding of ATP to the K_{ATP} channel, the channel shuts, causing membrane depolarization ($\Delta\Psi$), which thereby initiates the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (VDCC). Consequently, $[Ca^{2+}]_i$ rises and triggers the exocytosis of insulin. Several additional signals have been proposed to participate in metabolism-secretion coupling, as indicated. Depolarisation and increased $[Ca^{2+}]_i$ activate voltage-gated potassium channels (K_v) and Ca^{2+} -activated potassium channels (K_{Ca}), respectively, leading to an K^+ efflux from the β -cell and membrane repolarisation. Ca^{2+} is cleared from the cytosol by the action of the sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump, plasma membrane Ca^{2+} -ATPase (PMCA), and Na^+/Ca^{2+} exchanger (NCX), as well as by uptake into mitochondria and cytoplasmic Ca^{2+} -buffering proteins. The traces depicted (right) are adapted from (Henquin, Nenquin et al. 2009), and demonstrate the temporal and quantitative correlation between the membrane potential, $[Ca^{2+}]_i$ response and insulin secretion. Glucokinase (GK), glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), phosphofructokinase (PFK), fructose 1,6-bisphosphate (FBP).

The molecular mechanism of the amplifying pathway remains unknown. In recent years, many factors have been proposed to be involved including glutamate, malonyl-CoA, protein

phosphatases, PLC, protein kinases such as PKC or PKA, nucleotides such as GTP, cGMP, ATP, cAMP and NADPH, GTP-binding proteins, cAMP-guanosine nucleotide exchange factor 2 (Epac2), the transcription factor NF- κ B and H₂O₂ (Maechler and Wollheim 2001; MacDonald, Joseph et al. 2005; Pi, Zhang et al. 2010).

Islets lacking K_{ATP} channels because of knockout of either of the genes that encode the K_{ATP} channel subunits (SUR1 and Kir6.2) also display an operational amplifying pathway and can also secrete insulin upon glucose stimulation via this (K_{ATP} channel-independent) mechanism (Szollosi, Nenquin et al. 2007; Ravier, Nenquin et al. 2009).

Glucose-stimulated insulin secretion follows a biphasic time course *in vivo* and *in vitro*. In isolated islets the first-phase is characterised by a robust peak in insulin secretion which is followed by a second sustained phase of secretion (Fig. 1.2). Insulin secretion from isolated islets in the presence of continuous glucose elevation is pulsatile with a periodicity of 4–6 min (Tengholm and Gylfe 2009). The reason for this phenomenon is not fully understood but it has been observed that periodic fluctuations of insulin are pivotal to the action of the hormone on its target tissues by, for example, preventing downregulation of its receptors (Hellman 2009). Loss of pulsatile insulin release may contribute to insulin resistance and is an early indicator for T2DM (Lang, Matthews et al. 1981; Matthews, Naylor et al. 1983). Pulsatile insulin secretion from the pancreas requires coordination of the secretory activity between individual islets via intrapancreatic nerves and diffusible factors, as well as between individual β -cells via electrical coupling (gap junctions) and paracrine signals (Santos, Rosario et al. 1991; Tengholm and Gylfe 2009).

Pulsatile secretion from an individual islet is believed to occur due to the coordinated oscillations in both the metabolic and electrical activity of the individual β -cells within the islets (Gilon, Shepherd et al. 1993; Hellman 2009). Although the exact nature of these oscillations has not yet been elucidated, the M-type isoform of the glycolytic enzyme phosphofructokinase

(PFK-M) as well as changes in the intracellular concentrations of ATP and Ca^{2+} seem to play a crucial role (Bertram, Sherman et al. 2007).

PFK-M has a key influence on metabolism, which is mediated by a positive feedback effect of its product, fructose 1,6-bisphosphate (FBP), on the kinase activity and subsequent depletion of substrate (Tornheim 1997). The effects of ADP and ATP on K_{ATP} channels serve to couple metabolism to electrical activity of the β -cell (Ashcroft, Harrison et al. 1984). Ca^{2+} has both stimulatory and inhibitory feedback effects on metabolism and is also able to influence the plasma membrane potential by acting on Ca^{2+} -dependent K^+ channels (Gopel, Kanno et al. 1999). Ca^{2+} has an inhibitory effect on metabolism by decreasing ATP concentration through ATP-demanding clearance of Ca^{2+} from the cytosol (Chen, Koh et al. 2003). Ca^{2+} is also taken up into mitochondria, which leads to depolarisation across the mitochondrial inner membrane and thus reduces the driving force for ATP production (Bertram, Sherman et al. 2007). Conversely, Ca^{2+} positively influences metabolism by stimulating mitochondrial dehydrogenases (Bertram, Sherman et al. 2007; Tengholm and Gylfe 2009).

1.4 DIABETIC COMPLICATIONS AND REACTIVE OXYGEN SPECIES

T2DM can have a devastating impact on whole-body physiology. It is often associated with renal failure, blindness or nerve damage and is believed to be an important factor in the aetiology of heart attacks and strokes (Brownlee 2001). Tissue damage is especially common in cells that are unable to reduce their glucose uptake when exposed to hyperglycemia. This includes capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, neurons and Schwann cells in peripheral nerves and the β -cell itself. As the level of glucose rises, so the normal route of glucose metabolism becomes saturated, leading to activation of alternative pathways. These alternative glucose metabolism pathways exert their damaging effect on tissues by, for example,

producing toxic metabolites, inflammatory responses, modified transcription factors, and pathologic changes in gene expression. Interestingly, reactive oxygen species (ROS) are involved in insulin resistance (Houstis, Rosen et al. 2006). They are also implicated in most of the diabetic complications and are thought to play a vital role of the progression of the disease (Brownlee 2005).

Reactive oxygen species are chemical species formed by the incomplete reduction of oxygen. ROS include oxygen radicals - an atom or molecule that contains one or more unpaired electrons - such as the superoxide ($O_2^{\bullet -}$) or hydroxyl radical ($HO^{\bullet}$) and non-radical derivatives of oxygen such as hydrogen peroxide (H_2O_2) (Fig. 1.3). ROS can act as both oxidizing and reducing agents, and they are normally quite non-specific with regard to the type of molecule they react with.

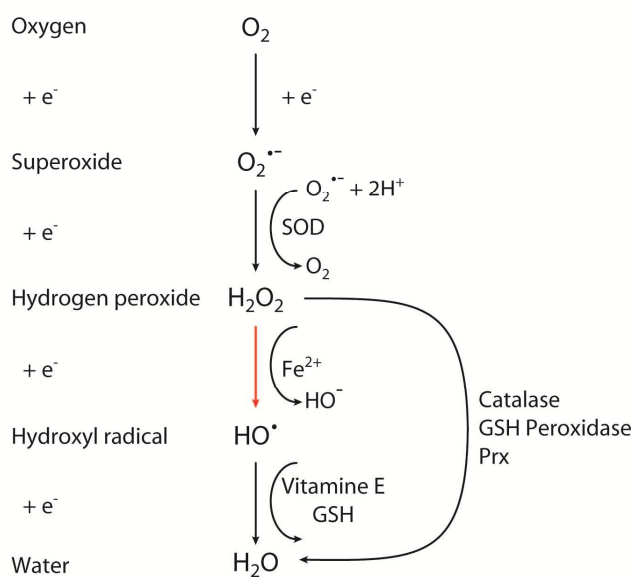


Fig. 1.3 Different forms of ROS and their detoxification by antioxidant defence enzymes and antioxidants

Oxygen accepts one electron to become superoxide which is quickly transformed to hydrogen peroxide by the enzyme superoxide dismutase (SOD). Hydrogen peroxide is enzymatically reduced to water by catalases, glutathione (GSH) peroxidases or peroxiredoxin (Prx). In the Fenton reaction (indicated by the red arrow), Fe^{2+} catalyses the one electron reduction of hydrogen peroxide to the very reactive hydroxyl radical. Small molecular antioxidants such as vitamin E or GSH can detoxify hydroxyl radicals.

The exceptions are $O_2^{\bullet-}$ and H_2O_2 , which are less reactive and highly specific for their targets. This makes them ideal mediators of signal transduction (Halliwell and M.C. Gutteridge 2007).

1.4.1 MULTIPLE PATHWAYS OF ROS PRODUCTION IN THE B-CELL ARE ACTIVATED BY CHRONIC GLUCOSE EXPOSURE

There are many metabolic sources of ROS inherent in normal cellular processes and the amount of ROS generated in the cell increases with its metabolic rate. With an ample nutrient supply, as found in diabetes, β -cells that respond to the prevailing hyperglycemia with enhanced secretion of insulin are at particular risk of accumulating large amounts of ROS. This ROS production is further increased by a positive feedback mechanism in which ROS inactivates glyceraldehyde 3-phosphate dehydrogenase and thereby promotes ROS-generating metabolic pathways (Brownlee 2005). Several ROS-producing pathways are known (Fig. 1.4).

The polyol pathway consumes the reducing equivalent NADPH, which is needed for regenerating intracellular antioxidants such as glutathione (GSH) or thioredoxin, and thereby indirectly increases the amount of ROS (Brownlee 2005). The hexosamine pathway increases H_2O_2 levels by a yet unknown mechanism (Kaneto, Xu et al. 2001). The PKC pathway can activate NADPH oxidase (NOX) (Uchizono, Takeya et al. 2006) which generates $O_2^{\bullet-}$ from O_2 .

Advanced glycation end products (AGE) are produced under aerobic conditions when glycated proteins react irreversibly with O_2 (Brownlee 2005). AGE cause oxidative stress by, for example, reacting with proteins in the electron transport chain, or molecules of the antioxidant defence system (Halliwell and M.C. Gutteridge 2007). Abnormal concentrations of glucose can also induce the production of thioredoxin-interacting protein (TXNIP) which binds to thioredoxin, an important electron donor for the antioxidant defence system, and inhibits its function. In turn this will lead to increased formation of ROS (Chen, Saxena et al. 2008).

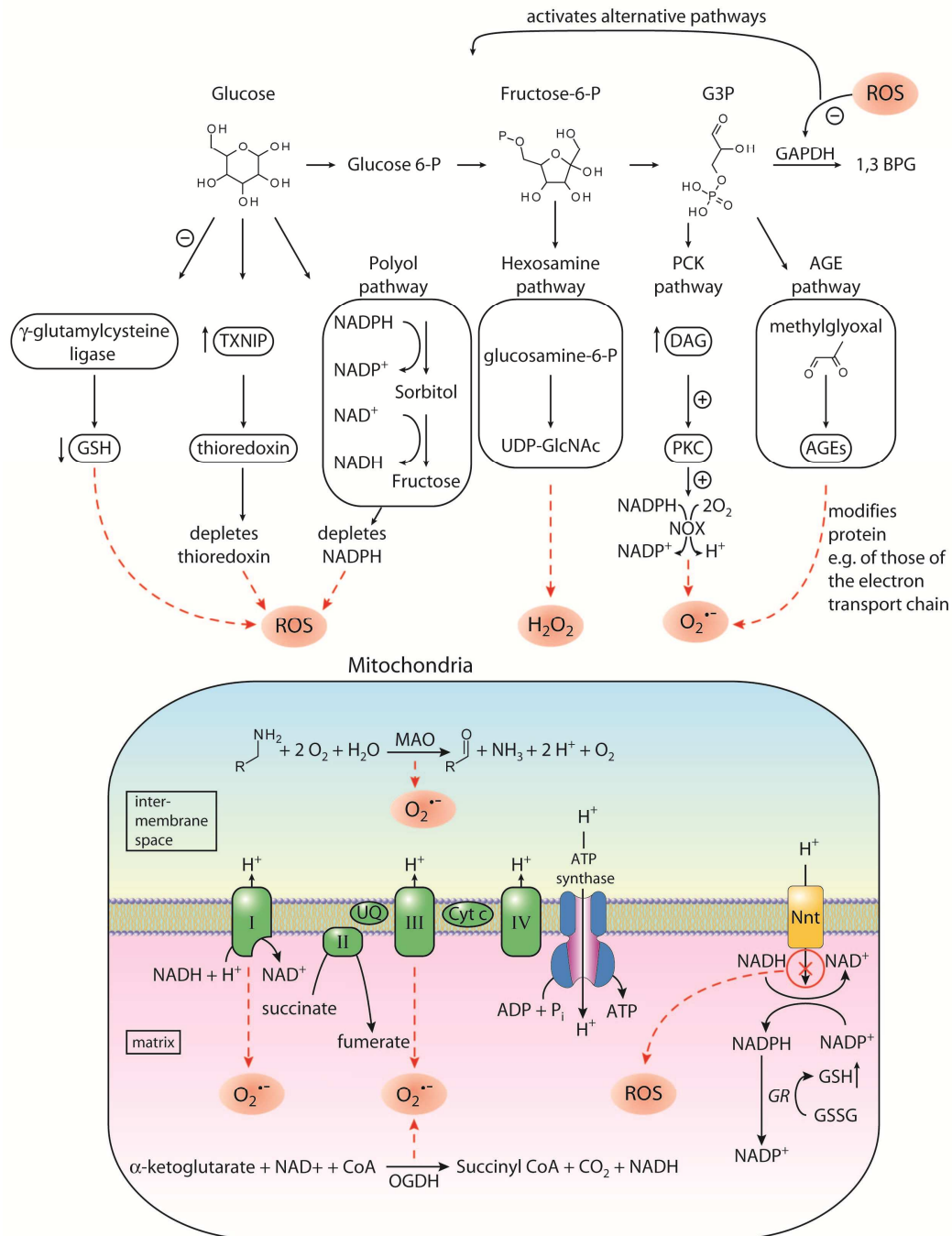


Fig. 1.4 Sources of ROS in the β -cell

ROS generated by the electron transport chain (in green) constitutes the main source of cellular ROS. In the mitochondria, ROS is also produced by monoamine oxidase (MAO) and α -ketoglutarate dehydrogenase (OGDH) or accumulates due to the lack of functional nicotinamide nucleotide transhydrogenase (Nnt). In the cytosol, alternate glucose metabolic pathways or toxic metabolites produce various forms of ROS either directly or indirectly by depleting antioxidant substances. Reduced glutathione (GSH), oxidised glutathione (GSSG), glutathione reductase (GR), thioredoxin-interacting protein (TXNIP), uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), glyceraldehyde 3-phosphate (G3P), diacylglycerol (DAG), protein kinase C (PKC), NADPH oxidase (NOX), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 1,3-biphosphoglycerate (1,3 BPG), advanced glycation end product (AGE).

Another compromising effect of long-term exposure to glucose on the antioxidant defence system is mediated by the inhibition of γ -glutamylcysteine ligase, the rate-limiting enzyme for glutathione synthesis (Catherwood, Powell et al. 2002).

Most cellular ROS, however, is generated by the electron transport chain of mitochondria. Metabolism of glucose generates the reducing equivalents NADH and FADH_2 via glycolysis and the TCA cycle. In the mitochondrial electron transport chain, electron transfer from NADH and FADH_2 to O_2 is then used to produce a proton gradient across the inner mitochondrial membrane. This electrochemical gradient provides the energy to make ATP from ADP by proton movement through the ATP synthase complex. When the mitochondrial electron transport chain is operational, 1-3 % of electrons are believed to escape, mainly in complexes I and III (Turrens 2003) and react with O_2 to form $\text{O}_2^{\bullet-}$, even in non-pathological states (Boveris 1977). There are several conditions under which an increase in $\text{O}_2^{\bullet-}$ generation can be observed:

- 1) high levels of glucose that cause an increase in the mitochondrial membrane potential;
- 2) a low supply of ADP, which causes back up of the electron flow,
- 3) an increase in mitochondrial Ca^{2+} which exerts a stimulatory effect on many enzymes involved in the TCA cycle or oxidative phosphorylation (Fridlyand and Philipson 2004; MacDonald, Joseph et al. 2005).

There are also other sources of ROS outside the electron transport chain. These include monoamine oxidase which generates $\text{O}_2^{\bullet-}$ in the outer mitochondrial membrane and alpha-ketoglutarate dehydrogenase which generates $\text{O}_2^{\bullet-}$ in the matrix (Halliwell and M.C. Gutteridge 2007). The lack of functional nicotinamide nucleotide transhydrogenase (Nnt) also leads to an accumulation of ROS. This is discussed further in paragraphs 1.6 and 3.1.1.

1.5 CELLULAR EFFECTS OF ROS

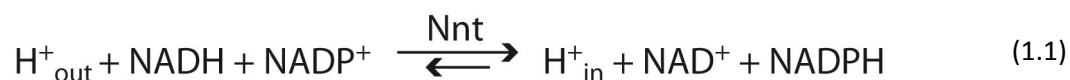
Under normal physiological conditions, the production of ROS is balanced by antioxidant defences. These include antioxidant enzymes such as superoxide dismutase, catalase, GSH peroxidase or peroxiredoxin and antioxidants such as vitamins, GSH, glutaredoxin or thioredoxin which detoxify ROS or act to repair oxidative damage on proteins and lipids (Halliwell and M.C. Gutteridge 2007; Mari, Morales et al. 2009). See Fig. 1.3. The cellular response to ROS depends on the severity and concentration of ROS. Many cells react to mild oxidative stress with increased proliferation, and if the ROS challenge increases, adapt by upregulation of their defence system (Halliwell and M.C. Gutteridge 2007). When ROS evades the antioxidant defence system it can cause structural and functional damage to proteins, lipids, carbohydrate or DNA mostly mediated by the very reactive hydroxyl radical. Secondary damage results from ROS-related changes such as ion levels or activation of stress-sensitive intracellular signalling pathways such as NF- κ B, p38 MAPK, JNK/SAPK (Newsholme, Haber et al. 2007) which can be part of the adaptive response or lead to apoptosis.

Adverse effects of ROS have been reported on insulin action (Evans, Maddux et al. 2005) and pancreatic islet function. The β -cell has weak antioxidant defence mechanisms, with low expression levels and activities of enzymes such as superoxide dismutase, catalase and glutathione peroxidase. This is considered to make the β -cell especially susceptible to ROS attacks, which will lead to β -cell dysfunction (Lenzen, Drinkgern et al. 1996; Tiedge, Lortz et al. 1997; Robertson 2006). ROS affects cellular processes resulting in a reduction of insulin content and secretion. This occurs by functional disruption of transcription factors such as Pdx-1 and MafA, which are critical for normal insulin gene expression (Harmon, Stein et al. 2005; Robertson 2006). Additional evidence that ROS has detrimental effects on insulin secretion is provided by studies in which it was shown that antioxidant treatment (Tanaka, Gleason et al. 1999) or increased

expression of antioxidant enzymes could protect the β -cell from dysfunction and ameliorate hyperglycemia (Tanaka, Tran et al. 2002).

1.6 NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE

Nicotinamide nucleotide transhydrogenase (Nnt) is a nuclear-encoded transmembrane protein, located in the inner mitochondrial membrane of all cells in the body (Arkblad, Egorov et al. 2002). Nnt catalyses the transfer of protons between NADH and NADPH (Fig. 1.4). Under normal physiological conditions (equation. 1.1), when the mitochondrial membrane potential is high, Nnt generates NADPH in the mitochondrial matrix (Hoek and Rydstrom 1988).



where the subscripts *out* and *in* refer to the inter-mitochondrial membrane space and the mitochondrial matrix, respectively. NADPH is an important reducing equivalent for sustaining redox homeostasis and preventing oxidative stress. It is a substrate for antioxidant enzymes such as glutathione reductase and thioredoxin reductase, thereby allowing regeneration of GSH and reduced thioredoxin, respectively (Nordberg and Arner 2001; Koehler, Beverly et al. 2006). GSH and thioredoxin are, in turn, used by GSH-peroxidases and peroxiredoxin, respectively to reduce H_2O_2 to water. The important role of Nnt in ROS detoxification is highlighted by a study on *C. elegans* which demonstrated that ablation of Nnt renders *C. elegans* more susceptible to oxidative stress and impairs the cellular ratio of reduced to oxidised glutathione (GSH/GSSG) (Arkblad, Tuck et al. 2005).

Studies of the inbred laboratory mouse strain C57BL/6J revealed that a loss-of-function mutation in Nnt is linked to glucose intolerance and reduced insulin release (Toye, Lippiat et al.

2005; Freeman, Shimomura et al. 2006), and demonstrated for the first time that ROS could cause glucose intolerance directly. It has been proposed that non-functional Nnt leads to ROS-mediated uncoupling of mitochondrial metabolism and consequently to a reduction in ATP synthesis and insulin secretion (Freeman, Shimomura et al. 2006). This thesis explores this hypothesis further.

1.7 THE ATP-SENSITIVE POTASSIUM (K_{ATP}) CHANNEL

The K_{ATP} channel is expressed throughout the body and is involved in numerous physiological processes in multiple cell types, including skeletal muscle, visceral and vascular smooth muscle, neurones and neuroendocrine cells (Akrouh, Halcomb et al. 2009). In the pancreatic β -cell, the K_{ATP} channel plays a key role in insulin secretion by coupling β -cell metabolism to the electrical activity of the plasma membrane (Ashcroft 2005). Opening of the channel prevents insulin secretion whereas closure induces insulin secretion. Consequently, mutations in the K_{ATP} channel may either prevent channel opening at low glucose, resulting in dysregulated insulin secretion and hypoglycemia (e.g. congenital hyperinsulinemia), or impede channel closure at high glucose, resulting in insufficient insulin secretion and diabetes (e.g. neonatal diabetes).

The K_{ATP} channel forms an octameric complex (Fig. 1.5) consisting of four pore-forming inwardly rectifying potassium channel subunits (Kir6.x) and four regulatory sulphonylurea receptors SURx subunits (Clement, Kunjilwar et al. 1997). Kir channels have cytosolic N and C termini and two transmembrane domains (TMDs), linked by a P-loop that inserts into the membrane to form the selectivity filter. The two subtypes of Kir6.x are Kir6.1, which is encoded by the gene *KCNJ8* and predominates in smooth muscle, and Kir6.2, which is encoded by the gene *KCNJ11* and forms the pore-forming subunit in pancreatic β -cells, neurons and cardiac cells. SUR is a member of the ATP-binding cassette transporter family (Aguilar-Bryan, Nichols et al. 1995). It contains 17 transmembrane helices organised in three domains (TMD0, TMD1 and TMD2) with

five, six and six helices, respectively. Two intracellular loops contain consensus sequences for nucleotide binding and hydrolysis and are termed nucleotide-binding domains (NBD1 and NBD2). The SUR subunit is encoded by the genes *ABCC8* (SUR1) and *ABCC9* (SUR2); the latter can be spliced into different forms to produce SUR2A and SUR2B (Aguilar-Bryan, Nichols et al. 1995; Chutkow, Simon et al. 1996). SUR1 is expressed in the pancreas and brain, whereas SUR2A is expressed in cardiac and skeletal muscle and SUR2B in various tissues including brain and smooth muscle (Ashcroft 2005).

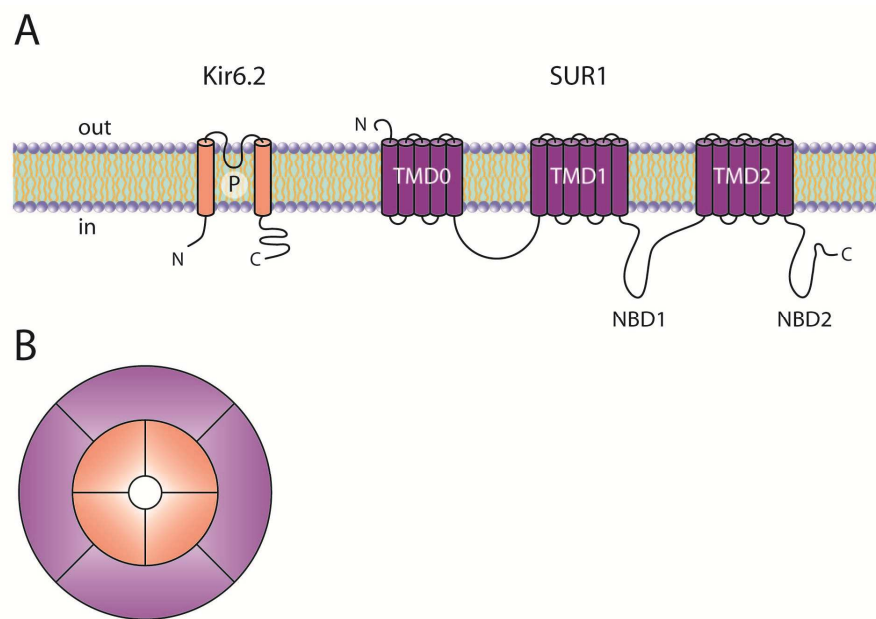


Fig. 1.5 K_{ATP} channel composition

A Transmembrane topology of a Kir6.2 and a SUR1 subunit. The two transmembrane helices of the Kir6.2 subunit are connected by a P-loop which forms the selectivity filter of the channel. SUR1 is composed of three sets of transmembrane domains (TMD0, TMD1 and TMD2) and contains two cytosolic nucleotide-binding domains (NBD1 and NBD1) involved in activation of the channel. **B** The octameric K_{ATP} channel complex is made up of four central Kir6.2 subunits, which form the channel pore, and four associated SUR subunits.

1.7.1 REGULATION OF K_{ATP} CHANNEL ACTIVITY

The regulation of the K_{ATP} channel is complex and can be influenced by numerous factors and cytosolic substances including phosphorylation, phosphatidylinositol phosphates and long-chain

acyl-CoA esters (Fan and Makielski 1997; Gribble, Proks et al. 1998; Tammaro 2009). However, in the pancreatic β -cell adenine nucleotides, produced by glucose oxidation, are regarded as the primary metabolic regulators of the channel (Tarasov, Girard et al. 2006).

The main physiological stimulus to K_{ATP} channel closure is the binding of ATP to the Kir6.2 subunit. Both MgATP and MgADP can activate the channel by interaction with the SUR1 subunit (Nichols, Shyng et al. 1996; Gribble, Tucker et al. 1998). However, MgATP can only exert its activating properties after being hydrolyzed to MgADP. Thus, it is the balance between ATP inhibition and MgATP and MgADP activation that determines the activity of the channel. The SUR1 subunit is also targeted by two different classes of drugs (Gribble and Reimann 2002). Sulphonylureas, like tolbutamide or glibenclamide, inhibit the channel and are used to treat T2DM. Opening of the K_{ATP} channel is produced by K_{ATP} channel openers, such as diazoxide, which is used to counteract hypersecretion of insulin in hyperinsulinemia (Inagaki, Gono et al. 1996).

1.7.2 NON-B-CELL MEDIATED EFFECTS OF THE K_{ATP} CHANNEL ON BLOOD GLUCOSE HOMEOSTASIS

K_{ATP} channel activity also exerts an effect on glucose homeostasis in various cell types and tissues other than the pancreatic β -cell. Secretion of the glucose-increasing hormone glucagon from pancreatic α -cells involves paracrine signals from β -cells (e.g. insulin, GABA or Zn^{2+} ions) or δ -cells (somatostatin), neural signals and intrinsic mechanisms (Gromada, Franklin et al. 2007). The K_{ATP} channel is expressed in α -cells and it has been proposed that it is directly implicated in glucose-sensing and the release of glucagon (MacDonald, De Marinis et al. 2007). In contrast to that of β -cells, the α -cell K_{ATP} channel exhibits much lower activity in the absence of glucose (Gopel, Kanno et al. 2000). This low K_{ATP} channel activity is thought to cause the spontaneous firing of action potentials, which leads to Ca^{2+} entry and stimulation of glucagon secretion at low glucose (Rorsman, Salehi et al. 2008). A rise in glucose would then close K_{ATP} channels further, leading to

an increase in membrane depolarisation. However, this depolarising stimulus causes inactivation of voltage-dependent ion channels (e.g. tetrodotoxin-sensitive Na^+ -channels or N-type Ca^{2+} -channels), that participate in the generating of the action potential and consequently decreases glucagon secretion. It has also been shown that insulin and Zn^{2+} secreted from β -cells can act on K_{ATP} channels in the α -cell in a paracrine fashion (Franklin, Gromada et al. 2005). Franklin et al. showed that both insulin and Zn^{2+} activate K_{ATP} channels in rat α -cells, hyperpolarising the plasma membrane and inhibiting electrical activity and glucagon release.

The muscle K_{ATP} channel (composed of Kir6.2 and SUR2A subunits) seems to play a role in the glucose uptake into skeletal muscle produced by insulin (Miki and Seino 2005). Studies on mice lacking muscle K_{ATP} channels due to knockout of either Kir6.2 (Miki, Nagashima et al. 1998) or SUR2A (Chutkow, Samuel et al. 2001) found that glucose uptake was enhanced. This suggests that closure of K_{ATP} channel enhances, whereas opening decreases, glucose uptake. However, the mechanisms by which K_{ATP} channel activity influences glucose uptake remain elusive.

K_{ATP} channels expressed in the central and peripheral nervous system are also involved in glucose homeostasis (Miki and Seino 2005). Evans et al. showed that closure of K_{ATP} channels in the brain (ventromedial hypothalamus) by sulphonylureas suppressed secretion of adrenaline and glucagon, two powerful hormones that raise glucose levels, leading to hypoglycaemia (Evans, McCrimmon et al. 2004). It was also demonstrated that opening of K_{ATP} channels in the mediobasal hypothalamus lowers blood glucose levels through inhibition of hepatic gluconeogenesis and, conversely, that closure of these channels results in hyperglycaemia (Pocai, Lam et al. 2005). Pro-opiomelanocortin (POMC)-expressing neurones play a central role in the hypothalamic regulation of satiety and appetite. Studies have shown that K_{ATP} channel activation in mouse POMC neurons leads to membrane hyperpolarisation and electrical silencing (Plum, Ma et al. 2006). This was associated with an abnormally increased appetite (hyperphagia) and weight gain. POMC neurons have also been shown to be excited by glucose (Ibrahim, Bosch et al. 2003).

In this study it was demonstrated that glucose depolarises the plasma membrane of POMC neurons and increases their firing rate in response to increases in extracellular glucose. Similar to the pancreatic β -cell, glucose sensing in POMC neurons is mediated by the K_{ATP} channel, which couples glucose metabolism to the electrical activity of the cell (Parton, Ye et al. 2007). When a gain-of-function mutation, which reduces but does not abolish electrical activity, was introduced into the POMC K_{ATP} channel, Parton et al. found that glucose sensing was impaired and mice became glucose intolerant (Parton, Ye et al. 2007).

1.8 AIM OF THE STUDY

This thesis uses mouse models and cell culture techniques to study genetic factors leading to disorders of insulin secretion such as diabetes or hyperinsulinemia (HI).

In Chapter 3 the role of reactive oxygen species (ROS) on glucose-stimulated increase in $[Ca^{2+}]_i$ and insulin secretion was investigated in three different ways. Firstly, the accumulation of ROS resulting from the knockdown of nicotinamide nucleotide transhydrogenase (Nnt) in MIN6 cells was examined. Secondly, the possible role of glutathione peroxidase 1 (GPx1) as a candidate gene for mitochondrial ROS detoxification, and its effect on insulin secretion, was investigated. Thirdly, the effect of exogenous GSH on the glucose-stimulated $[Ca^{2+}]_i$ response in (1) Nnt mutant and (2) control islets was tested.

In Chapter 4 transgenic mouse models of neonatal diabetes, carrying an activating mutation (V59M) in the Kir6.2 subunit of their K_{ATP} channels, were characterised. In particular, the rise in $[Ca^{2+}]_i$ triggered by glucose or tolbutamide was analysed in islets isolated from wild-type and transgenic mice expressing the V59M mutant either constitutively or in an inducible fashion.

In Chapter 5 a knock-in mouse model of hyperinsulinemia of infancy (HI), which expresses an inactivating mutation (E1506K) in the SUR1 subunit of the K_{ATP} channel, was investigated. Specifically, the insulin secretion profile, Ca^{2+} signalling and electrophysiological properties of SUR1 E1506K mutant islets were characterised.

2 GENERAL METHODS

2.1 ANIMAL CARE

All experiments were conducted in accordance with the 1986 UK Animals (Scientific Procedures) Act and University of Oxford ethical guidelines, and all animal studies were approved by the Ethics Committee of the University of Oxford. Mice were housed in same-sex littermate groups of 2 to 8 in a temperature- and humidity-controlled room on a 12-hour light/12-hour dark cycle (lights on at 6 am). Regular chow food (Teklad Global 2019 Rodent containing 55% carbohydrate, 19% protein, and 9% fat; Harlan Teklad) was freely available except where it is indicated mice were fasted overnight. Mice had *ad libitum* access to water at all times.

2.2 BUFFERS/MEDIA

2.2.1 PANCREATIC ISLET ISOLATION AND SECRETION STUDIES

- Hank's Solution: 138 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 2.6 mM CaCl₂, 1 mM NaH₂PO₄, 4.2 mM NaHCO₃, 10mM HEPES, pH 7.4 with NaOH, fortified with 2.7 mM glucose and 1 mg/ml BSA on the day of experiment.

- Krebs Bicarbonate Buffer (KRB): 118.5 mM NaCl, 2.54 mM CaCl₂, 1.19 mM KH₂PO₄, 4.74 mM KCl, 25 mM NaHCO₃, 1.19 mM MgSO₄, 10 mM HEPES, pH 7.4 with NaOH, supplemented with 1 mg/ml BSA on the day of experiment

2.2.2 LIGHT MICROSCOPIC IMAGING AND FLUOROMETRY

- EC Buffer-I: 137 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 2.6 mM CaCl₂, 10 mM HEPES, pH 7.4 with NaOH
- EC Buffer-I with 70 mM KCl: 67 mM NaCl, 70 mM KCl, 1.2 mM MgCl₂, 2.6 mM CaCl₂, 10 mM HEPES, pH 7.4 with NaOH

2.3 PANCREATIC ISLET ISOLATION AND SECRETION STUDIES

Mice were killed by cervical dislocation and islets released from surrounding tissue of the pancreas by injection of 2 ml Liberase (Roche) solution (0.5 mg/ml Hank's Solution) into the common bile duct (Szot, Koudria et al. 2007). Subsequently, the pancreas was removed and digested in Liberase solution for 20 min at 37°C. The islets were then hand-picked into a Petri dish with Hank's Solution. The procedure was repeated until the dish was free of tissue debris. Hand-picked islets were cultured for 24 hours in Pancreatic Islet Medium (Hcell) in a 37°C, 7% CO₂ incubator prior to starting experiments. This was to allow them to recover from the 'shock' of the isolation procedure.

2.3.1 INSULIN SECRETION ON PERIFUSED ISLETS

About 50 islets were placed on top of 8- μ m pore diameter filter membrane mounted inside a Swinnex filter holder chambers (Millipore) and perfused with KRB solution at 37°C at a rate of 1 ml/min. After 30 min of pre-perfusion, to equilibrate the islets, the experiment was started and 1 ml of samples were collected. The islets were first perfused for 10 min with 2 mM glucose KRB. Subsequently the perfusion solution was switched to one containing glucose and/or tolbutamide at the concentrations indicated in the text. Samples (5 μ l) of the collected perfusate were be assayed for insulin using a Mouse Insulin ELISA kit (Mercodia Ultrasensitive). At the end of the experiment, the insulin content of the islets was extracted by incubating the islets in 95% ethanol and 5% acetic acid at -20°C over night. Insulin was measured by ELISA.

2.4 LIGHT MICROSCOPIC IMAGING AND FLUOROMETRY IN PANCREATIC ISLETS

Measurements of $[Ca^{2+}]_i$ was performed on whole islets, because studying the behaviour of β -cells in intact islets has several advantages over single cell studies. Recordings on whole islets preserve the natural environment for β -cells. In intact islets, intercellular signalling can occur via both chemical (paracrine) and electrical signals (Rorsman, Berggren et al. 1989; Charollais, Gjinovci et al. 2000). Dispersing the islet into single cells disrupts those pathways and may explain why single β -cells show a greater heterogeneity in their response to glucose and also a much poorer insulin secretion than β -cells in whole islets (Herchuelz, Pochet et al. 1991; Luther, Hauge-Evans et al. 2006). Indeed, it was found that the electrophysiological properties of β -cells within the intact islet differ from those of isolated cells in aspects such as channel density and voltage dependence of their activation and inactivation (Gopel, Kanno et al. 1999; Zhang, Galvanovskis et al. 2008).

2.4.1 FLUOROMETRIC MEASUREMENTS OF $[Ca^{2+}]_i$ IN PANCREATIC ISLETS

To measure $[Ca^{2+}]_i$ in pancreatic islets the ratiometric Ca^{2+} probe fura 2 was used. The dye was introduced into the islets at room temperature by incubation in 3 μ M of the lipophilic, cell-permeant acetoxymethyl ester (fura 2 AM, Invitrogen), dissolved in EC Buffer-I. Loading was done at RT temperature in order to avoid compartmentalisation of the dye into intracellular organelles which may occur at physiological temperatures (Molecular Probes 2008). The non-ionic detergent pluronic acid used at a dilution of 0.01% was added to the incubation buffer to facilitate solubilisation and uptake of fura 2 AM. After 20 min, an islet was transferred to a perfusion chamber mounted on the stage of an Axiovert 200 microscope (Zeiss) where it was held in place by a glass pipette through suction. To allow for complete de-esterification of intracellular AM ester and to remove external dye, the islet was perfused with warm EC Buffer-I (33-35°C) for 15 min. During imaging, the islet was continuously perfused with warm EC buffer (33-35°C), containing different glucose concentrations or tolbutamide, as indicated.

We used a fluorescence system (Photon Technology International) for ratiometric measurements with dual excitation at 340 nm and 380 nm, and recorded emission at 510 nm. Pancreatic islets were imaged at 2 frames per sec and fluorescence intensity (F) was detected with a photomultiplier tube. Ca^{2+} signals are presented as the ratio of F at different excitation wavelengths (F340/F380). Data were analyzed using FeliX32 Analysis software. The experiment was designed so that sample and control islets were examined on one day. Experiments were repeated (with the same numbers of islets) and the data of islets with the same genotype and treatment were pooled into one group for analysis.

2.4.1.1 DETERMINATION OF THE MAXIMAL $[Ca^{2+}]_i$ INCREASE OR DECREASE RATE

To determine the maximal increase rate of change in $[Ca^{2+}]_i$ upon glucose or tolbutamide stimulation, the Hill equation 1 (equation 2.1) was fitted to various parts of the Ca^{2+} trace and the obtained fitted function was differentiated.

$$y = a1 + \frac{a2 - a1}{1 + \left(\frac{a3}{x}\right)^{a4}} \quad (2.1)$$

When islets were incubated with glibenclamide their basal $[Ca^{2+}]_i$ was elevated. In these islets, glucose stimulation produced a transient fall in $[Ca^{2+}]_i$ which returned to levels similar to basal $[Ca^{2+}]_i$. To measure the maximal decrease rate of the transient fall in $[Ca^{2+}]_i$ upon glucose stimulation, the Hill equation 2 (equation 2.2) was fitted to various parts of the Ca^{2+} trace and the obtained fitted function was differentiated.

$$y = a2 + \frac{a1 - a2}{1 + \left(\frac{a3}{x}\right)^{a4}} \quad (2.2)$$

The constants a1, a2, a3 and a4 were used as fitting parameters. a1 is the bottom plateau. a2 is the top plateau. a3 is the time (x) at which the ratio (y) is halfway between a1 and a2. a4 is the Hill factor, describing the steepness of the curve. Fitting and differentiation was performed in MATLAB with the help of an in-house programme written by Dr Juris Galvanovskis.

2.4.2 CONFOCAL CALCIUM IMAGING

For confocal Ca^{2+} imaging, islets were loaded with 4 μ M fluo 4-AM (Invitrogen) for 2 hours at room temperature in EC Buffer-I. Islets were then placed in a specimen chamber on the stage of a Zeiss Axioskop 2 FS MOT microscope. They were allowed to equilibrate for about 15 min while being continuously perfused with EC Buffer-I at 37°C. The islets were held in place by a glass

suction pipette. Changes in $[Ca^{2+}]_i$ were recorded using a confocal laser scanning system (Zeiss LSM 510 META). Individual cells were selected as 'regions of interest' with the LSM software, and their $[Ca^{2+}]_i$ responses to the various stimuli were identified as changes in fluo 4 emission intensity at 500–550 nm upon excitation with the 488 nm line of an argon laser. To avoid phototoxicity and to reduce bleaching of the dye, the laser energy was kept at a low output level (3-5%) and the acquisition of images was set to 3 sec intervals.

Ca^{2+} imaging was used to study the oscillatory behaviour of $[Ca^{2+}]_i$ in single β -cells within the intact islets and to investigate the number of β -cells responding to glucose stimulation. β -cells were identified by their $[Ca^{2+}]_i$ response to show a robust elevation of $[Ca^{2+}]_i$ to stimulation with extracellular glucose (Nadal, Quesada et al. 1999). The $[Ca^{2+}]_i$ response was expressed as the percentage of F/F_0 , where F is the recorded fluorescence and F_0 the initial fluorescence observed in the absence of stimulus.

2.5 STATISTICS

The data are expressed as means $\pm$ standard error of the mean (SEM). Statistical significance was investigated using a two-tailed t-test for unpaired or paired data as indicated. For comparisons of the means of more than two independent groups, a one-way ANOVA was used. Two-way ANOVA was used to analyse the effect of two factors such as genotype and drug treatment on a measured variable ($[Ca^{2+}]_i$ response or HyPer fluorescence). Post tests following a one- or two-way ANOVA was performed using the Bonferroni method. Means were deemed significantly different if the p-value was less than or equal to 0.05. All statistical tests were performed using the software SPSS, Excel or GraphPad Prism.

3 ROLE OF REACTIVE OXYGEN SPECIES IN INSULIN SECRETION

3.1 INTRODUCTION

3.1.1 NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE: ITS ROLE IN B-CELL METABOLISM

Previous studies of the inbred laboratory mouse strain C57BL/6J revealed that a loss-of-function mutation in the gene encoding nicotinamide nucleotide transhydrogenase (Nnt) causes glucose intolerance and reduced first- and second-phase insulin release compared with strains that express wild-type Nnt (Toye, Lippiat et al. 2005; Freeman, Shimomura et al. 2006). Conversely, transgenic expression of a wild-type Nnt gene in C57BL/6J mice increased insulin secretion and improved glucose tolerance (Freeman, Hugill et al. 2006). Similarly, Freeman et al. showed that silencing the Nnt gene by siRNA in insulin-secreting cells caused impaired mitochondrial ATP synthesis, and thereby failure of glucose-dependant K_{ATP} channel closure and insulin secretion (Freeman, Shimomura et al. 2006). In an independent study, glucose-stimulated hypersecretion of insulin in DBA/2 mice was associated with increased glucose metabolism and ATP levels. This phenotype was mainly attributed to the increased expression and activity of Nnt (Aston-Mourney, Wong et al. 2007). Therefore, Nnt may be considered a strong candidate gene for type 2 diabetes.

It has been hypothesised that loss of Nnt function is linked to an increase in mitochondrial ROS and leads to a disruption of insulin secretion. In addition to the deleterious chemical modification of bio-molecules discussed above, ROS may also evoke short-term physiological reactions and activate uncoupling protein 2 (UCP2).

Activation of UCP2 (Fig. 3.1), a carrier protein located in the inner mitochondrial membrane, facilitates increased proton flux into the mitochondrial matrix which depolarises the inner mitochondrial membrane potential and leads to a reduction in ATP synthesis (Krauss, Zhang et al. 2003). In insulin-producing cells enhanced UCP2 activity, and the concomitant fall in ATP levels, results in decreased insulin secretion (Chan, De Leo et al. 2001; Zhang, Baffy et al. 2001).

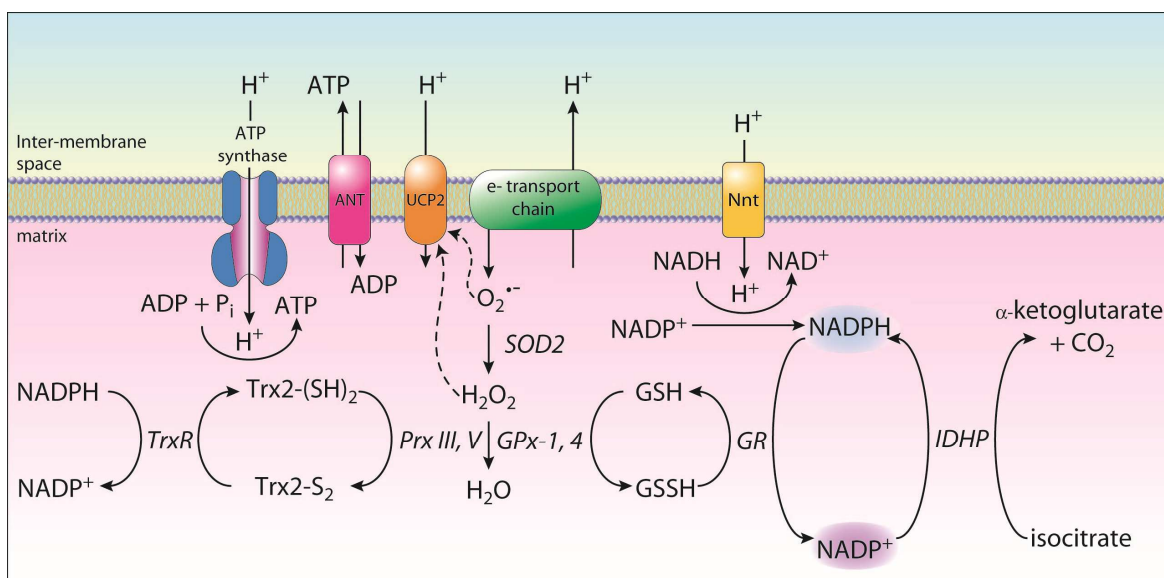


Fig. 3.1 Postulated mechanism of the function of Nnt in insulin secretion

Reactive oxygen species (ROS) in the form of superoxide ($O_2^{\bullet-}$) are generated as a by-product of the electron transport chain. $O_2^{\bullet-}$ can be converted to hydrogen peroxide (H_2O_2), in a reaction catalysed by Superoxide Dismutase 2 (SOD2). Under normal physiological conditions, when the inner mitochondrial membrane is hyperpolarized, Nnt and NADP⁺-dependent isocitrate dehydrogenase (IDHP) are the main source of NADPH in the mitochondrial matrix. NADPH is used as a reducing equivalent by glutathione reductase (GR) and thioredoxin reductase (TrxR) to provide reduced glutathione (GSH) and thioredoxin ($Trx2-(SH)_2$). The mitochondrially expressed peroxidases, glutathione peroxidases 1 and 4 (GPx-1, 4) and peroxiredoxin III and V (Prx III, V) reduce H_2O_2 to H_2O by oxidizing TrxR and GSH respectively. Thus, when Nnt is non-functional, ROS increases, leading to activation of uncoupling protein 2 (UCP2), dissipation of the mitochondrial membrane potential and reduced ATP synthesis. A low ATP concentration, in turn, leads to a decrease in insulin secretion.

It has been hypothesised that when Nnt is non-functional, mitochondrial NADPH is low and removal of ROS is impaired. ROS accumulation is expected to activate UCP2, leading to reduced ATP synthesis and consequently reduced insulin release. In order to test this hypothesis I measured ROS (in the form of H_2O_2) inside the mitochondria of Nnt-silenced cells. Additionally, I investigated the effects of GSH on glucose-stimulated Ca^{2+} influx in islets isolated from homozygous N68K or G745D Nnt mutant mice. N68K or G745D are point mutations that render the Nnt protein non-functional (Freeman, Shimomura et al. 2006). I also investigated whether reduced glutathione is able to restore normal Ca^{2+} influx in these mutant islets. Finally, I investigated the role of the ROS-detoxifying enzyme glutathione peroxidase 1 (GPx1) on insulin secretion.

3.1.2 GLUTATHIONE PEROXIDASE 1 (GPx1): ITS ROLE IN INSULIN SECRETION

It has been shown that ROS production in insulin-producing cells leads to a reduction in insulin secretion (Tanaka, Tran et al. 2002; Moriscot, Richard et al. 2003). By overexpressing GPx1 in INS-1 cells (Moriscot, Richard et al. 2003) or whole pancreatic rat islets (Tanaka, Tran et al. 2002) the impaired insulin secretion can be partially restored. Transgenic mice overexpressing GPx1 showed hyperinsulinemia and increased β -cell mass *in vivo*, and hypersecretion of insulin measured in isolated islets (Wang, Vatamaniuk et al. 2008). These changes were associated with an upregulation of the transcription factor Pdx-1: this protein is involved in β -cell differentiation, insulin gene expression and synthesis, and a downregulation of UCP2 in islets.

GPx1 belongs to a family of glutathione peroxidases (GPxs) that are found in both prokaryotes and eukaryotes, including bacteria, fungi and animals (Margis, Dunand et al. 2008). The mammalian family of GPxs contains seven members, which exhibit distinct but overlapping tissue- and cellular-distributions, and substrate specificity (Brigelius-Flohe 1999; Utomo, Jiang et

al. 2004). GPxs catalyse the reduction of H_2O_2 or organic hydroperoxides (ROOH), such as fatty acid hydroperoxide, to water or the corresponding alcohols (ROH) by using mainly the thiol-containing tripeptide GSH as an electron donor (Fig. 3. 2).

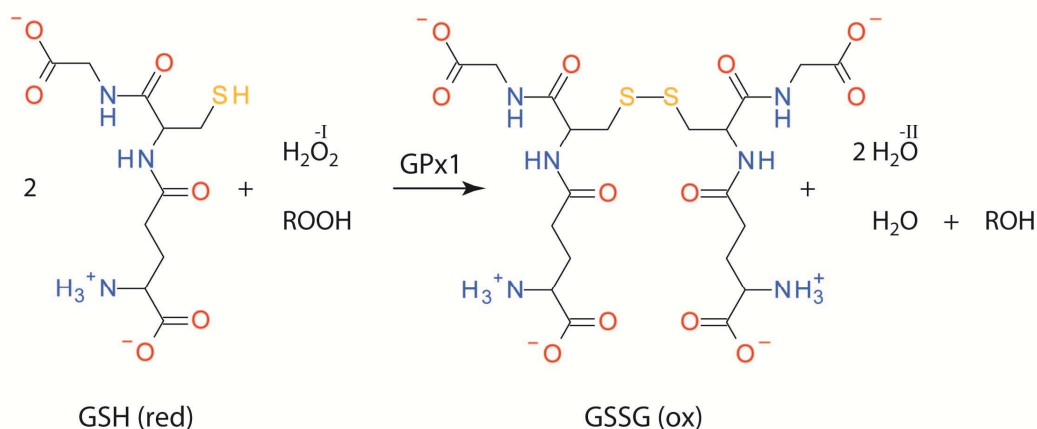


Fig. 3.2 Enzymatic reactions of glutathione peroxidase 1 (GPx1)

GPx1 catalysis the reduction of hydrogen peroxide (H_2O_2) or other organic hydroperoxides (ROOH) to water and corresponding alcohols (ROH). GPx1 utilizes reduced glutathione (GSH) as the electron donor for this redox reaction and oxidises GSH to GSSG.

GPx1 is ubiquitously expressed in all cells of the body and its expression is particular high in tissues with elevated oxidative stress such as erythrocytes, liver, kidney, or lung (Brigelius-Flohe 1999; Lei, Cheng et al. 2007) and low in pancreatic β -cells (Grankvist, Marklund et al. 1981). GPx1 is mainly found in the cytosol but also found in the nucleus and mitochondria (Panfili, Sandri et al. 1991; Esworthy, Ho et al. 1997; Legault, Carrier et al. 2000; Chang, Cho et al. 2004; Mari, Morales et al. 2009; Miranda, Wang et al. 2009).

Supporting evidence for the role of GPx1 in the removal of mitochondrial H_2O_2 in β -cells was provided by Wang et al. They demonstrated that the increase in insulin secretion caused by overexpression of GPx1 is associated with an increase in mitochondrial membrane potential ($\Delta\Psi_m$) and reduced expression of UCP2 (Wang, Vatamaniuk et al. 2008). This suggests GPx1 is part of the Nnt pathway and responsible for the detoxification of H_2O_2 in β -cell mitochondria. This

hypothesis would imply that failure to detoxify H_2O_2 will lead to H_2O_2 accumulation, activation of UCP2 and decreased ATP production. This would eventually result in reduced insulin secretion (Fig 3.1). In order to test this hypothesis, I used RNA interference to silence the GPx1 gene in MIN6 cells and measured the effect on mitochondrial H_2O_2 and glucose-stimulated insulin secretion.

3.2 METHODS

3.2.1 BUFFERS/MEDIA

3.2.1.1 PROTEIN BIOCHEMISTRY

- Blocking Buffer: 50 mM Tris, 138 mM NaCl, 2.7 mM KCl, 0.05% TWEEN® 20, pH 8.0 + 3% milk
- Protein Sample Buffer: 1 part NuPAGE® LDS Sample Buffer + 1 part of RIPA Buffer
- Washing Buffer: 50 mM Tris, 138 mM NaCl, 2.7 mM KCl, 0.05% TWEEN® 20, pH 8.0
- RIPA Buffer: 150 mM NaCl, 50 mM Tris-HCl pH 7.6, 0.1% SDS, 1% NP-40, 1% triton, 2 mM EDTA, 1% Sodium deoxycholate, supplemented with protease inhibitor cocktail (Complete, EDTA free, by Roche)

3.2.1.2 CELL CULTURE

- Dulbecco's Phosphate Buffered Saline (DPBS), without calcium chloride and magnesium chloride (Sigma-Aldrich)
- Dulbecco's Modified Eagle Medium (DMEM) (1X) liquid (high glucose), containing GlutaMAX I, 4500 mg/l glucose and 110 mg/l sodium (Invitrogen). DMEM was supplemented with 15% Foetal Bovine Serum (FBS, Invitrogen) and a 1:100 dilution of Penicillin-Streptomycin (Invitrogen)
- TrypLE Select (Invitrogen)

3.2.1.3 INSULIN SECRETION OF MIN6 CELLS

- Krebs Bicarbonate Buffer (KRB): 118.5 mM NaCl, 2.54 mM CaCl₂, 1.19 mM KH₂PO₄, 4.74 mM KCl, 25 mM NaHCO₃, 1.19 mM MgSO₄, 10 mM HEPES, pH 7.4 with NaOH, supplemented with 1 mg/ml BSA on the day of experiment

3.2.1.4 MOLECULAR BIOLOGY

- LB Agar: LB Broth medium, 20 g/l agar
- LB Broth medium: 10 g/l Tryptone, 5 g/l Yeast Extract, 10 g/l NaCl

3.2.1.5 LIGHT MICROSCOPIC IMAGING AND FLUOROMETRY

- EC Buffer-I: 137 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 2.6 mM CaCl₂, 10 mM HEPES, pH 7.4 with NaOH
- EC Buffer-I with 70 mM KCl: 67 mM NaCl, 70 mM KCl, 1.2 mM MgCl₂, 2.6 mM CaCl₂, 10 mM HEPES, pH 7.4 with NaOH

3.2.2 MOLECULAR BIOLOGY

3.2.2.1 GENOTYPING OF C57BL/6J MICE

Genotypes were identified by PCR using genomic DNA isolated from ear biopsies (DNeasy Blood and Tissue Kit; QIAGEN). C57BL/6J mice were genotyped by PCR of the missing exons 7 and 11 of the Nnt gene. Primer sequences for Nnt exon 7 are: NntXn7 forward: GTGCATTGAACCCTCAAAGG

and NntXn7 reverse: CAGGTAAGAAAGCTCCTGTTTT; for Nnt exon 11: NntXn11 forward TCCTGCTATTCCTCCTCCTG and NntXn11 reverse GCTGCCTTGACTTTGGATATT. The following PCR protocol was applied to yield amplicons for exon 7 of ~ 200 bp and exon 11 of ~ 250 bp: 10 min at 94°C followed by 37 cycles of 94°C for 20 sec, 49°C for 1 min, then 72°C for 1 min, followed by a final extension 72° for 5 min.

3.2.2.2 ESTIMATION OF DNA AND RNA CONCENTRATION

DNA or RNA concentration was determined using a spectrophotometer (NanoDrop® ND-1000). The sample was pipetted onto the measurement pedestal and in the 'Nucleic Acid' application module, the concentration of DNA or RNA was calculated by the Beer-Lambert equation (eq. 3.1):

$$c = \frac{A \times e}{b} \quad (3.1)$$

where c is the nucleic acid concentration in ng/μl, A is the absorbance in Absorbance units (AU), e is the wavelength-dependent extinction coefficient in ng x cm/μl and b is the path length in cm. The default extinction coefficients used by the programme are: 50 ng x cm/μl for double-stranded DNA and 40 ng x cm/μl for RNA. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. A ratio of ~ 1.8 was accepted as pure for DNA; a ratio of ~ 2.0 was accepted as pure for RNA.

3.2.2.3 TRANSFORMATION OF DH5A E.COLI AND PLASMID ISOLATION

Competent DH5 α *E.coli* were thawed on ice. 1 μl of ice cold plasmid DNA was mixed with the cells and incubated for 30 min on ice. Cells were then heat-shocked for 45 sec at 42°C. Subsequently, 900 μl of warm LB broth medium was added to the cells, which were then shaken

at 250 rpm for 1 hour at 37°C. 50 µl of medium was then spread onto a LB agar plate containing the appropriate selection antibiotic (30 µg/ml Kanamycin for the pHyPer-dMito vector) and grown overnight at 37°C. Single colonies were picked and used to grow a 100 ml LB Broth medium culture overnight, containing the appropriate selection antibiotic. The plasmid was purified using a HiSpeed Plasmid Purification Kit (Qiagen) according to manufacturer's instruction.

3.2.2.4 RNA INTERFERENCE

RNA interference (RNAi) is a natural biological defence mechanism used by a variety of organisms to destroy foreign double-stranded RNA molecules such as those found in viruses. RNAi constitutes a powerful experimental technique for inducing potent inhibition of gene expression.

3.2.2.5 SHORT INTERFERING RNA

Short interfering RNAs (siRNAs) are 21-23 nucleotide long double-stranded RNA molecules which facilitate the cleavage and degradation of mRNA strands that carry a complementary sequence. siRNAs were designed and purchased from Ambion / Applied Biosystems. The siRNA sequences were not disclosed upon purchase. The Nnt gene was silenced with siRNA ID: 63421. Control cells consisted of cells transfected with siRNA bearing no homology to known gene sequences (Silencer® Negative Control #1 siRNA; Part Number AM4611). These control siRNAs were termed nonsense siRNA (ns-siRNA). Each siRNA had a Cy3 tag on the 5' end of the sense strand to enable visualization of transfected cells by fluorescence with a TRITC (Rhodamine)/DiI/Cy3 filter set. Cells were incubated with > 50 nM siRNA for over 24 hours to produce silencing of the target gene.

3.2.2.6 SHORT HAIRPIN RNA

Short hairpin RNA (shRNA) is an RNA molecule that contains of a sense strand, an antisense strand, and a short loop sequence between the sense and antisense fragments. Due to the complementarity of the sense and antisense fragments in their sequence, such RNA molecules tend to form hairpin-shaped double-stranded RNA. shRNA sequence was cloned into a DNA vector, allowing for expression from a pol III type promoter. The expressed shRNA is then exported into the cytoplasm where it activates the silencing machinery.

shRNA has the advantage over siRNA that transfected cells can be selected using antibiotic resistance transferred by the vector. This makes long-term stable gene silencing possible. MISSION® shRNAs vectors were used to silence the GPx1 gene. These were obtained from Sigma-Aldrich as bacterial glycerol stocks. Single colonies were recovered as described by the manufacturer's instruction. Plasmid DNA was then purified from 500 ml of selective LB medium (+ 100 µg/ml carbenicillin) cultures using the HiSpeed® Plasmid Purification Kit (Qiagen). Among the five clones tested the clone Mm00656767_g1 with the sequence CCGGCCGGGACTACACCGAGATGAACTCGAGTTCATCTCGGTGTAGTCCCGGTTTTTG proved to show the greatest success of knockdown. Cells transfected with the nonsense shRNA (ns-shRNA) vector (MISSION® TRC2 Control Vector pLKO-puro Non-Target shRNA), which activates the RNAi pathway but does not target any known genes, were used as control cells.

3.2.2.7 RELATIVE QUANTIFICATION OF GENE KNOCKDOWN USING THE TAQMAN® GENE EXPRESSION ASSAYS

The TaqMan Gene Expression Assay is based on the PCR technique. It exploits the 5' → 3' exonuclease activity of Taq DNA polymerase to quantitatively appraise gene expression (Fig. 3.3).

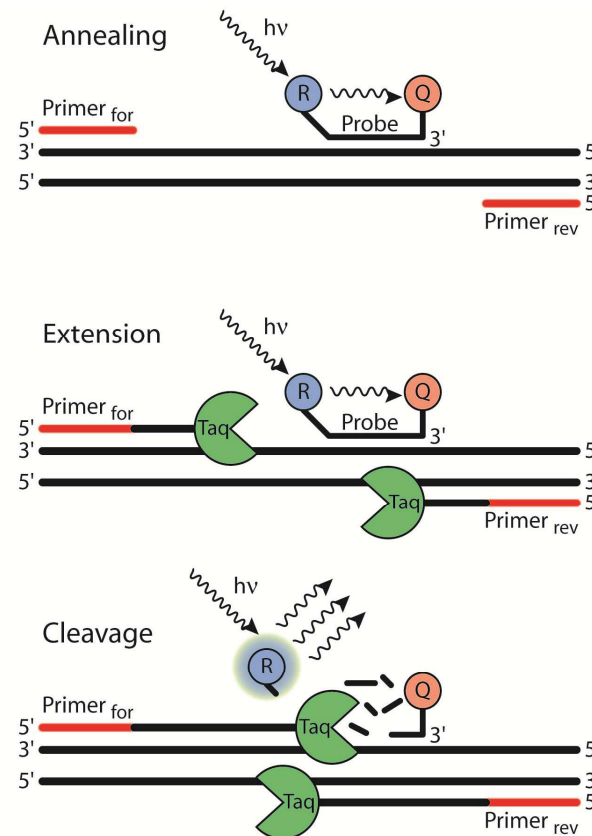


Fig. 3.3 The TaqMan® Gene Expression Assay

Two primers are designed to be specific for the gene of interest. A third oligonucleotide, the probe, is hybridised to a nucleotide sequence between the two primers. The probe is conjugated to a reporter dye (R) at the 5' end and a quencher dye (Q) at its 3' end. As *Taq* DNA polymerase (Taq) synthesizes a new DNA strand primed by the forward primer, the enzyme approaches the probe and its 5' → 3' exonuclease activity processively degrades the probe. Thereby, reporter dye and quencher dye are separated, which results in increased fluorescence ($h\nu$) of the reporter. An increasing fluorescence signal can be related to an accumulation of PCR products and the amount of starting material.

The process can be divided into three steps. In the first step, RNA is isolated from cells, bearing the gene of interest. In the second step, cDNA is reverse transcribed from total RNA. In the final PCR step, PCR products are synthesized from cDNA. The PCR step in the TaqMan Gene Expression Assay also contains a third primer, called a probe, which binds specifically to a site on the target sequence between the forward and a reverse primer. The probe is labelled with two fluorophores. A reporter dye (R) is attached at the 5' end, and a quencher dye (Q) is attached at its 3' end, which also renders it unsuitable for priming new DNA synthesis. Due to the proximity of

the reporter dye to the quencher dye, fluorescence is suppressed by Förster resonance energy transfer (FRET). During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5'→3' exonuclease activity of the DNA polymerase cleaves the probe, causing release of separate nucleotides containing the dye and the quencher. The separation of reporter and quencher leads to an increase in fluorescence signal and can be correlated to accumulation of the PCR product.

3.2.2.8 TAQMAN PCR: EXPERIMENTAL PROCEDURES

3.2.2.8.1 RNA ISOLATION

Total RNA was isolated from cells using an RNeasy® Mini Kit (Qiagen). The sample was processed according to the manufacturer's instruction. An additional on-column DNase-I digestion (Qiagen) was carried out.

3.2.2.8.2 REVERSE TRANSCRIPTION

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize single-stranded cDNA. The reaction was performed according to the manufacturer's manual.

3.2.2.8.3 PERFORMING PCR AMPLIFICATION AND ANALYSIS

In the final step, the TaqMan Universal PCR Master Mix (2 X) (Applied Biosystems) was used to amplify the target sequence. The PCR reaction was performed in MicroAmp™ Optical Plates (Applied Biosystems) and run on an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). Pre-designed primers and probes were obtained from Applied Biosystems as

inventoried Assays. Primer and probe sequences are proprietary. To exclude genomic DNA from the amplification, a probe was chosen to span exon-exon boundaries – if possible. The TaqMan primes used are listed in table 3.1:

gene	Assay ID Details
GPx1	Mm00656767_g1
beta-2 microglobulin (B2M)	Mm00437764_m1
Nnt	Mm00435154_m1
Gapdh	Mm99999915_g1

Tab. 3.1 TaqMan primers used in the experiments

A mathematical model for relative quantification in real time PCR is presented by Pfaffl et al. (Pfaffl 2001). In short, the amount of cDNA encoding the target gene (i.e. Nnt or GPx1) in a given sample, and thereby indirectly the amount of correspondent mRNA, is determined as an n-fold difference relative to the expression of target gene in control cells (i.e. cells treated with control siRNA or shRNA vector). The relative expression ratio of a target gene is then normalised to the expression of an unregulated reference gene transcript (B2M or Gapdh) to take account of differences in the amount of total RNA added to each reaction.

This relative expression ratio (R) can be described by the following equation (3.2).

$$R = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta CT_{\text{ref}}(\text{control-sample})}} \quad (3.2)$$

where CT is the cycle number at which the fluorescence rises above the background fluorescence.

$\Delta CT_{\text{target}}$ refers to the difference in the CT values of the target gene (Nnt or GPx1) measured in

control cells (cells treated with ns-siRNA or ns-shRNA vector) and sample cells (cells treated with siRNA or shRNA directed against Nnt or GPx1). ΔCT_{ref} is the difference in CT values of the reference gene (B2M or Gapdh) recorded in control cells and sample cells. E_{target} and E_{ref} are the amplification efficiencies of the target and reference genes, respectively. These efficiencies have to be determined experimentally by performing a standard curve with a series of, for example, two-fold dilutions of cDNA. Equation (3.3) describes the exponential amplification of PCR products, with X_n = the number of molecules at cycle n , X_0 = the initial number of molecules, E = the efficiency of amplification, and n = number of cycles.

$$X_n = X_0 E^n \quad (3.3)$$

Transformation of equation (3.3) into a logarithmic form, and solving it for n , yields equation (3.4).

$$n = -\frac{1}{\lg E} \times \lg X_0 + \frac{\lg X_n}{\lg E} \quad (3.4)$$

It can be seen that plotting CT values versus the log of the input cDNA gives the slope of the straight line as slope = $-1/\lg E$, with $E = 10^{(-1/\text{slope})}$.

3.2.3 CELL CULTURE TECHNIQUES

3.2.3.1 MAINTENANCE OF MIN6 CELLS

MIN6 cells were grown in DMEM medium equilibrated with 7% CO₂ and 93% air at 37°C. Cells were passaged every three to five days by aspirating the supernatant and washing the cells with DPBS. Having dissociated the cells with TrypLE Select, they were spun down at ~ 300 g for 2.5 min and resuspended in fresh medium. Subsequently, cells were plated at a density of approximately 2.5×10^5 cells/cm². MIN6 cells used in the present study were harvested at passages 20-30.

3.2.3.2 TRANSFECTION OF siRNA INTO MIN6 CELLS

MIN6 cells were plated in Fluoro Dishes™ (World Precision Instruments) or 6 well plates (Nunc) at a density of 5×10^5 cells per Fluoro Dish or well, 24 hours prior to transfection. The transfection was performed using Lipofectamine™ 2000 (Invitrogen). On the day of transfection, the medium was aspirated, and the cells were washed with DPBS. Cells were then incubated at 37°C in 7% CO₂ and 93% air with 1 ml siRNA-Lipofectamine complex. This complex was prepared according to the manufacturer's protocol using an amount of siRNA sufficient for knockdown of the gene, in 1 ml of DMEM without supplement and 5 µl of Lipofectamine. After 4-6 hours, the solution was topped up with 1.5 ml of DMEM. Cells were used for experiments 24 hours after transfection.

3.2.3.3 TRANSFECTION OF THE MISSION® shRNA INTO MIN6 CELLS AND SELECTION OF TRANSFECTED CELLS

$8-6 \times 10^6$ cells were plated into a 80 cm² cell culture flask and transfected with 19 µg of shRNA vector DNA and 57 µl TransIT®-LT1 (Mirus Bio) according to the manufacturer's protocol.

3.2.3.4 PUROMYCIN TITRATION

The MISSION® shRNAVector endows transfected cells with a puromycin resistance gene. To determine the lowest concentration of puromycin needed to efficiently select transfected cells, a puromycin titration was performed. Cells (5×10^5) were plated into wells in a 25-well plate with 500 µl fresh media. The next day, 500-10000 ng/ml of puromycin was added to each well. The minimum concentration of puromycin (3 µg/ml) that caused complete cell death after 2-3 days was used. Cell viability was determined by a propidium iodide exclusion assay. When incubated with DMEM containing 50 µM propidium iodide, dead cells take up the dye and incorporate it

into their DNA causing it to fluoresce upon excitation at 535 nm (emission was measured at 617 nm). A TRITC filter set on an Axiovert microscope (Zeiss) was used for detection.

3.2.3.5 TRANSFECTION OF HyPER PLASMID DNA INTO MIN6 CELLS AND GENERATION OF STABLE CELL LINES

Lipofectamine™ 2000 (Invitrogen) was used to transfect MIN6 cells with the hydrogen peroxide-sensitive probe HypPer. A culture of 150×10^5 cells in an 80 cm² flask was transfected 32 µg of HyPer DNA and 80 µl of Lipofectamine in 20 ml of DMEM. After 4-6 hours, the transfection media was removed and replaced with 20 ml of DMEM. To generate stably transfected clones, a selection marker (1.6 mg/ml G418 for HyPer selection) was added to the growth medium 24 hours after transfection. The selection medium was replaced every 5-7 days.

3.2.4 INSULIN SECRETION OF MIN6 CELLS

24 hours prior to the assay, MIN6 cells were plated into a 96-well plate at a density of $6-9 \times 10^4$ cells per well. On the day of the experiment, the cells were washed with DPBS. The solution was then replaced with KRB and the cells were incubated for 1 hour at 37°C. After aspiration of the KRB solution, insulin secretion was stimulated by adding 200 µl of various concentrations of glucose dissolved in KRB. Following 1 hour of incubation at 37°C, the plate was put on ice and the supernatant was collected. To determine the insulin content of the cells, the supernatant was fully aspirated and the cells were lysed by adding 200 µl of 95% ethanol 5% acetic acid and incubating at -20°C over night. The insulin concentration in the samples was measured according to manufacturer's instructions using a Mouse Insulin ELISA (Mercodia Ultrasensitive) with 5µl sample volume. Insulin secretion was expressed as a percentage of insulin content.

3.2.5 PROTEIN BIOCHEMISTRY

3.2.5.1 PREPARATION OF MIN6 LYSATE

Since GPx1 expression in MIN6 cells is very low, cells were directly lysed in Protein Sample Buffer and subjected to western blot analysis. In brief, cells (3×10^5) were washed in DPBS and then lysed in 45 μ l of Protein Sample Buffer. The sample was homogenized in a shredder column (Qiagen) by centrifugation at 17000 g for 2 min and then heated for denaturing electrophoresis at 70°C for 10 min.

3.2.5.2 PROTEIN QUANTIFICATION

Total protein was quantified using a detergent-compatible bicinchoninic acid (BCA) protein Assay Kit (Pierce) according to the manufacturer's instructions.

3.2.5.3 WESTERN BLOTTING

Protein samples were first separated according to their molecular weight by denaturing polyacrylamide gel electrophoresis (NuPAGE® Electrophoresis System by Invitrogen). Briefly, 3 parts of the solution containing the protein samples were diluted with 1 part of NuPAGE® LDS Sample Buffer (4 X) and heated at 70°C for 10 min. NuPAGE® Novex Bis-Tris Gels (gradient of 4-12% acrylamide) were loaded with 10-20 μ l of sample and 200 V was applied for 30 min. A MES SDS Running Buffer (NuPAGE®), containing Tris⁺, MES⁻, and dodecylsulfate ions, was used. The separated proteins were transferred to a nitrocellulose membrane (Whatman) using a XCell II Blot Module at 30 V for 1 hour and NuPAGE® Transfer Buffer with 20% methanol. Following a blocking step with Blocking Buffer for 1 hour, the membrane was probed with a primary antibody

(Tab. 3.2), raised against the antigen of interest, dissolved in Blocking Buffer. The primary antibody was incubated overnight at 4°C. After a subsequent washing (3x with Washing Buffer), the membrane was incubated for 1 hour at RT with a horseradish peroxidase (HRP) conjugated secondary antibody (Tab. 3.3) directed toward the primary antibody. Finally, unbound antibody was washed away and the immobilised protein was visualised using a chemiluminescent substrate (SuperSignal West Femto by Thermo scientific). The signal was captured on CL-XPosure Film (Thermo Scientific). For estimation of molecular weight the Spectra™ Multicolor Broad Range Protein Ladder (Fermentas) was used.

Antigen	species (raised)	type	dilution
Nnt, mouse, against sequence aa 1073-1086 (custom made by Eurogentec)	rabbit	polyclonal	1/650
actin, MAB1501 (Millipore)	mouse	monoclonal	1/1000
GPx1, mouse, ab22604 (Abcam)	rabbit	polyclonal	1/300
β-tubulin, mouse, 556321 (BD Pharmingen)	mouse	monoclonal	1/333

Tab. 3.2 Primary antibodies used in the experiments

Antigen	species (raised)	type	dilution
rabbit IgG, HRP conjugated, NA 934 (GE Healthcare)	donkey	polyclonal	
mouse IgG, HRP conjugated, NA 931 (GE Healthcare)	sheep	polyclonal	1/1000 (if anti actin 1. ab) 1/5000 (if anti β-tubulin 1. ab)

Tab. 3.3 Secondary antibodies (ab) used in the experiments

3.2.5.4 DENSITOMETRY

To quantify the intensity of bands on a Western blot, the gel analysis routine in the freeware programme ImageJ was performed. The relative protein expression (R_p) of a target gene (Nnt or GPx1) was calculated as the ratio of the density of the sample (cells treated with siRNA or shRNA) to that of the control (cells treated with ns-siRNA or ns-shRNA), normalised to the expression ratio of a reference gene (actin or β -tubulin). See equation (3.5).

$$R_p = \frac{\frac{sample_{target}}{control_{target}}}{\frac{sample_{ref}}{control_{ref}}} \quad (3.5)$$

Where R_p is relative protein expression ratio. Sample target and control target are the densities of the target gene measured in the sample and control respectively. Sample ref and control ref are the densities of the reference gene measured in the sample and control respectively.

3.2.6 LIGHT MICROSCOPIC IMAGING AND FLUOROMETRY

3.2.6.1 MITOCHONDRIAL HYDROGEN PEROXIDE MEASUREMENTS IN MIN6 CELLS USING HyPER

HyPer is a genetically encoded fluorescent protein capable of specifically detecting intracellular hydrogen peroxide (H_2O_2) (Belousov, Fradkov et al. 2006). It consists of a yellow fluorescent protein which has been inserted into the regulatory domain of the H_2O_2 -sensing transcription factor OxyR, expressed by *E. coli*. HyPer does not cause artificial ROS generation and can be used for detection of fast changes of H_2O_2 . When the appropriate signal peptide is attached, HyPer can be expressed in different cell compartments. In this thesis, I used the mitochondrial form of HyPer: pHyPer-dMito. HyPer displays two excitation peaks (420 nm and 500 nm) and one emission peak (516 nm). Upon exposure to H_2O_2 , the excitation peak at 420 nm decreases in

proportion to the increase at the excitation peak at 500 nm. These spectral characteristics render it suitable for ratiometric measurements, thereby eliminating false results caused by photobleaching or differences in HyPer expression.

Before imaging, cells were incubated in EC Buffer-I for 1 hour with 7% CO₂ and 93% air at 37°C. During the experiment, cells were continuously perfused with EC Buffer-I at 37°C and imaged on 35 mm Fluoro Dishes (World Precision Instruments) using an epifluorescence microscope (Nikon, Eclipse, TE 2000-U) with a CFI Super Fluor 40x/1.3 oil objective (Nikon). We used an IonOptix fluorescence system (Boston, Massachusetts) for ratiometric measurements with a dual excitation at 480/20 nm and 405/40 nm and emission at 530/40 nm (filters from Chroma Technology). Data were collected with a CCD camera (IonOptix). Cells were excited at one frame per three sec, or one frame per 10 sec for experiments lasting for 35 min.

3.2.6.2 ROS MEASUREMENTS IN MIN6 CELLS USING DICHLORODIHYDROFLUORESCIN DIACETATE

Before the experiment, cells were loaded for 25 min at 37°C with 10 µM of the ROS detection reagent *2',7'-dichlorodihydrofluorescein diacetate SE* (H₂DCF), dissolved in EC Buffer-I. This special derivative of H₂DCF contains an amine-reactive succinimidyl ester, which allows for covalent binding to intracellular components, thus permitting a longer retention within the cell. The oxidation of *2',7'-dichlorodihydrofluorescein diacetate, SE* by ROS was visualised with an excitation at 480/40 nm, and emission was collected using a 510 nm long pass filter.

3.2.6.3 MEASUREMENTS OF $[Ca^{2+}]_i$ IN MIN6 CELLS

MIN6 cells, attached to the bottom of a 35 mm Fluoro Dishes (World Precision Instruments), were loaded for 25 min at room temperature with 4 μ M fura 2 AM (Invitrogen), dissolved in EC Buffer-I. The cells were then mounted on the stage of an epifluorescence microscope (Nikon, Eclipse, TE 2000-U) where they were continuously perfused with EC Buffer-I at 37°C. After a resting period of 15 min to allow for complete de-esterification of intracellular AM ester and to remove external dye, cells were imaged with a CFI Super Fluor 40x/1.3 oil objective (Nikon). Different glucose concentrations or tolbutamide were applied through the perfusion system. We used an IonOptix fluorescence system (Boston, Massachusetts) for ratiometric measurements with a dual excitation at 340 nm and 380 nm, and recorded emission at 510 nm. Fluorescence was collected with a CCD camera (IonOptix). Cells were excited at one frame per three sec. Ca^{2+} signals were presented as the fluorescence ratio (F340/F380) at different excitation wavelengths.

3.2.7 $[Ca^{2+}]_i$ RESPONSES AND ANALYSED PARAMETERS IN PANCREATIC ISLETS

In order to analyse $[Ca^{2+}]_i$ responses of pancreatic islets quantitatively, various parameters were measured, as described in Fig. 3.4.

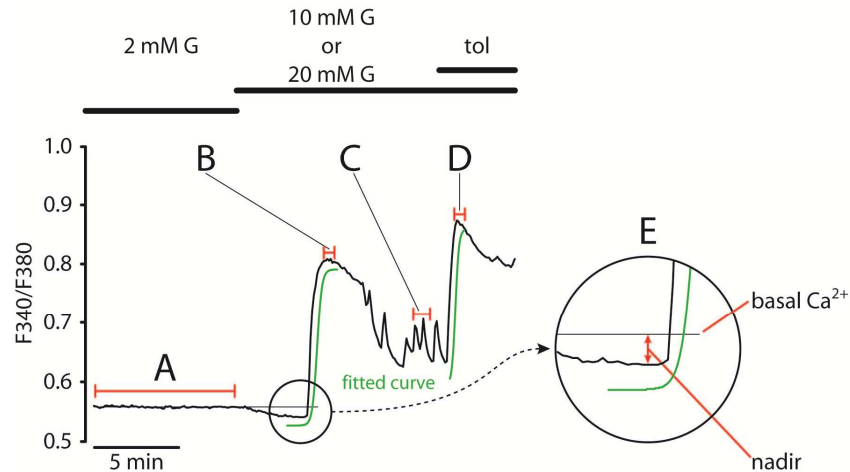


Fig. 3.4 Example of a representative $[Ca^{2+}]_i$ response recorded from a pancreatic islet stimulated with 10 or 20 mM glucose and 0.2 mM tolbutamide

The parameters analysed are shown. **A** Time over which basal $[Ca^{2+}]_i$ in 2 mM glucose was measured. **B** Time over which the peak $[Ca^{2+}]_i$ response, recorded in either 10 mM or 20 mM glucose as indicated in experiment, was measured. Approximately 60 data points, corresponding to a time interval of 30 sec, were averaged. **C** Time over which steady state $[Ca^{2+}]_i$ was determined. About 120 data points, corresponding to 1 min, were averaged. Measurements were either taken 5 min or 10 min (as indicated in the experiment) after the peak glucose response. **D** Time over which the peak $[Ca^{2+}]_i$ response in 0.2 mM tolbutamide (tol) was quantified. Approximately 60 data points, corresponding to a time interval of 30 sec, were averaged. Tolbutamide was either applied in the presence of 2 mM, 10 mM or 20 mM glucose as indicated in the experiment. **E** The nadir of initial fall in $[Ca^{2+}]_i$ (nadir) was calculated as the difference between basal Ca^{2+} , measured in A, and the minimum $[Ca^{2+}]_i$ before the glucose-stimulated Ca^{2+} peak. Experiments where no nadir was observed were excluded from the analysis. The Ca^{2+} increase upon glucose and tolbutamide stimulation (if present) was fitted to the Hill equation 1 (green lines). The fitted curves have been offset relative to the original data for clarity. Parameters for the Hill equation 1 are given in Chapter 2 (General Methods).

3.3 RESULTS

3.3.1 MEASUREMENT OF REACTIVE OXYGEN SPECIES GENERATED BY THE MITOCHONDRIAL ROS GENERATOR MENADIONE

Menadione causes increases in peroxide and superoxide radicals within the mitochondria by stimulating redox cycling in the electron transport chain (Halliwell and M.C. Gutteridge 2007). It has been shown that application of menadione (30 μ M) to mouse pancreatic acinar cells induces generation of ROS (Criddle, Gillies et al. 2006). In contrast, a high concentration of menadione (100 μ M over a 4 hour exposure) caused cell death in the rat pancreatic acinar cell line AR4-2J (Sata, Klonowski-Stumpe et al. 1997). In this experiment, I determined whether a low concentration of menadione (50 μ M) could elicit detectable ROS levels in the mouse insulin-secreting β -cell line MIN6 (Ishihara, Asano et al. 1993).

Since the strong photooxidation of the ROS detector dichlorodihydrofluorescein diacetate (H_2DCF) did not allow for continuous kinetic measurements, two incubation times for menadione were tested (30 min and 60 min) (Fig. 3.5). After 30 min, a significant increase in ROS was measured by H_2DCF . No membrane blebbing or other morphological signs of apoptosis were observed. However, H_2DCF failed to show any detectable changes in the level of ROS in response to glucose stimulation (data not shown).

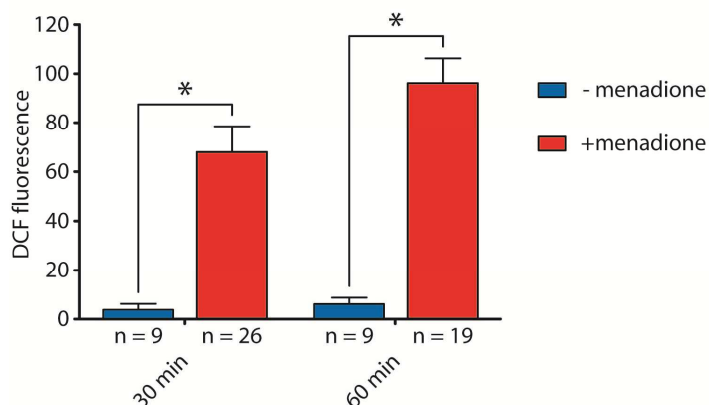


Fig. 3.5 Menadione increases ROS levels inside MIN6 cells

MIN6 cells were incubated with 50 μ M menadione for 30 min or 60 min. The level of ROS was measured as DCF fluorescence. The data were collected in two independent experiments and indicate the mean $\pm$ SEM of 9-25 cells as indicated. Significance was tested using an unpaired t-test. * $p < 0.05$.

3.3.2 MEASUREMENT OF MITOCHONDRIAL H_2O_2 IN MIN6 CELLS STABLY TRANSFECTED WITH HyPer

Commonly, ROS is detected using derivatives of H_2DCF , as described above. However, this dye has several serious disadvantages:

- 1) it is not specific and is sensitive to multiple types of ROS.
- 2) it cannot be targeted to specific intracellular compartments.
- 3) and most importantly, it can produce ROS upon exposure to light (Soh 2006).

HyPer is devoid of these disadvantages. It is a novel genetically encoded, protein-based fluorescent sensor capable of detecting intracellular hydrogen peroxide (Belousov, Fradkov et al. 2006). It can also be targeted to specific cellular compartments. I therefore examined whether menadione increases H_2O_2 within the mitochondria, using MIN6 cells and mitochondrially targeted HyPer.

In EC Buffer-I the Hyper signal was stable. It took about 1 min for menadione to reach the cell and produce an increase in fluorescence, corresponding to an increase in H_2O_2 (Fig. 3.6 A). The signal rose in a sigmoidal fashion and plateaued after approximately 7 min.

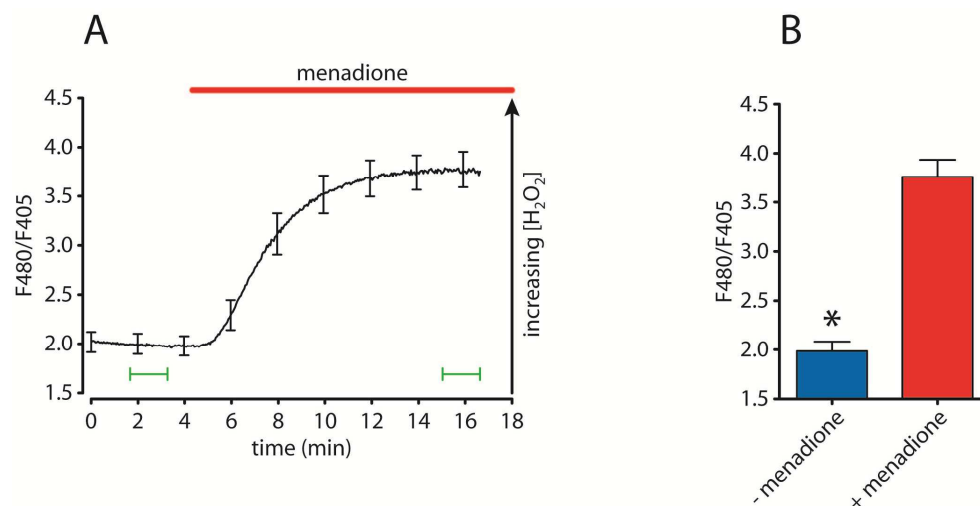


Fig. 3.6 Menadione increases the level of mitochondrial H_2O_2 in MIN6 cells detected by HyPer

A Average signal from 28 cells expressed as the ratio of 480 nm and 405 nm excitation. The SEM is shown for data points separated by a 2 min interval. The time course has been corrected for perfusion delay. **B** Steady state HyPer fluorescence, before (-) and after (+) menadione addition. Approximately 40 data points, corresponding to a time interval of 1.6 min (as indicated with green scale in A), were averaged. The data were collected in five dishes and indicate the mean $\pm$ SEM of $n=28$ cells. Significance was tested using a paired t-test. * $p < 0.05$

The mean HyPer fluorescence of MIN6 cells showed a clear and significant increase upon addition of the mitochondrial ROS generator (Fig. 3.6 B). Next, I investigated the effect of Nnt knockdown on the menadione mediated generation of ROS in MIN6 cells.

3.3.3 SUCCESSFUL NNT KNOCKDOWN WITH siRNA WAS VERIFIED BY (I) QUANTITATIVE PCR AND (II) WESTERN BLOT ANALYSIS

3.3.3.1 (I) QUANTITATIVE PCR

To investigate the functional role of Nnt in β -cells, siRNA was used to silence the Nnt gene in the MIN6 cells. MIN6 cells were used instead of native β -cells because they are more amenable to genetic modifications such as RNA interference or the expression of introduced plasmids. Control cells were transfected with nonsense siRNA (ns-siRNA), bearing no homology to known gene sequences.

Real-time PCR efficiencies (E) of the target gene (Nnt) and the reference gene (Gapdh) were calculated by plotting the CT values versus the cDNA concentration (expressed in a logarithmic form) and using the equation $E = 10^{(-1/\text{slope})}$ (Fig. 3.7 A; see Chapter 2 for further details of methods).

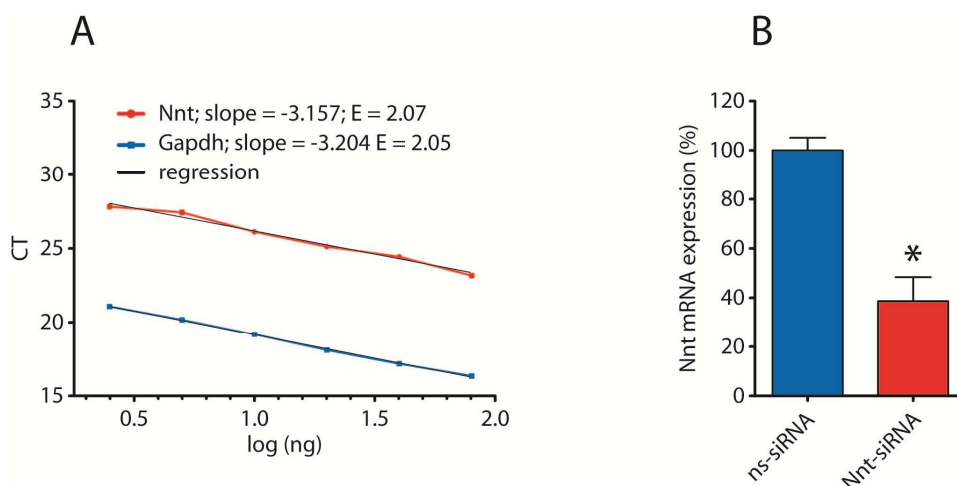


Fig. 3.7 Measurement of Nnt gene expression using quantitative PCR

A Determination of real-time PCR efficiencies of the target gene (Nnt) and reference gene (Gapdh). Data points were mean $\pm$ SEM of 3 PCRs. See methods for further detail. **B** Knockdown efficiency of Nnt recorded from MIN6 cell transfected with nonsense siRNA (ns-siRNA) and Nnt-siRNA. Data are the mean $\pm$ SEM of 3 independent experiments. Significance was tested using an unpaired t-test. * $p < 0.05$.

The PCR efficiencies were then used to determine the knockdown efficiencies of Nnt. siRNA against Nnt (Nnt-siRNA) led to an approximately 60% reduction in Nnt mRNA (Fig. 3.7 B). For the following experiments, only cells showing a knockdown of 60% or more were used. Under our conditions, about 95% of cells were transfected, as monitored by the fluorescence of a Cy3 fluorophore which was covalently linked to the siRNA. This indicates that a knockdown of 60% is maintained at the level of an individual cell as well as the whole population.

3.3.3.2 (II) WESTERN BLOT ANALYSIS

In ns-siRNA transfected cells, Western blotting with antibodies to Nnt (the target gene) or actin (the reference gene) revealed a single band at ~ 130 kDa and ~ 40 kDa, respectively (Fig. 3.8 A). Transfection with Nnt-siRNA reduced Nnt protein expression but left expression of actin unaltered. Quantification of Nnt protein expression in Nnt-siRNA treated cells showed a knockdown of about 60% in comparison to ns-siRNA transfected cells (Fig. 3.8 B), consistent with the quantitative PCR data.

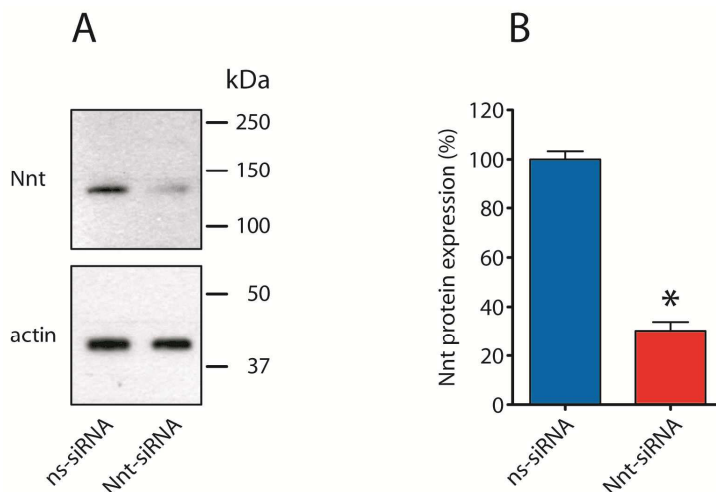


Fig. 3.8 Western blot analysis of ns-siRNA and Nnt-siRNA transfected MIN6 cells

A Each lane was probed with antibodies against Nnt and actin, as indicated. **B** Quantitative analysis of Western blots by densitometry. Data are mean $\pm$ SEM of 3 experiments. Significance was tested using an unpaired t-test. * $p < 0.05$.

3.3.4 MITOCHONDRIAL H₂O₂ MEASURED IN MIN6 NNT KNOCKDOWN CELLS UPON MENADIONE INCUBATION

In order to examine the role of Nnt in preventing ROS generation, MIN6 cells in which Nnt had been knocked down by siRNA were incubated with 50 μ M menadione (Fig. 3.9). Only cells that were positively identified from the presence of the Cy3 tag on the siRNA were imaged. Nnt-silenced MIN6 cells showed a faster and larger increase in mitochondrial H₂O₂ than ns-siRNA transfected cells (Fig. 3.9 A). The steady state fluorescence ratios showed that the lower increase in mitochondrial H₂O₂ produced by menadione in Nnt-silenced cells was extremely significant ($P < 0.0001$) compared to the effect of menadione on ns-siRNA transfected cells (Fig. 3.9 B).

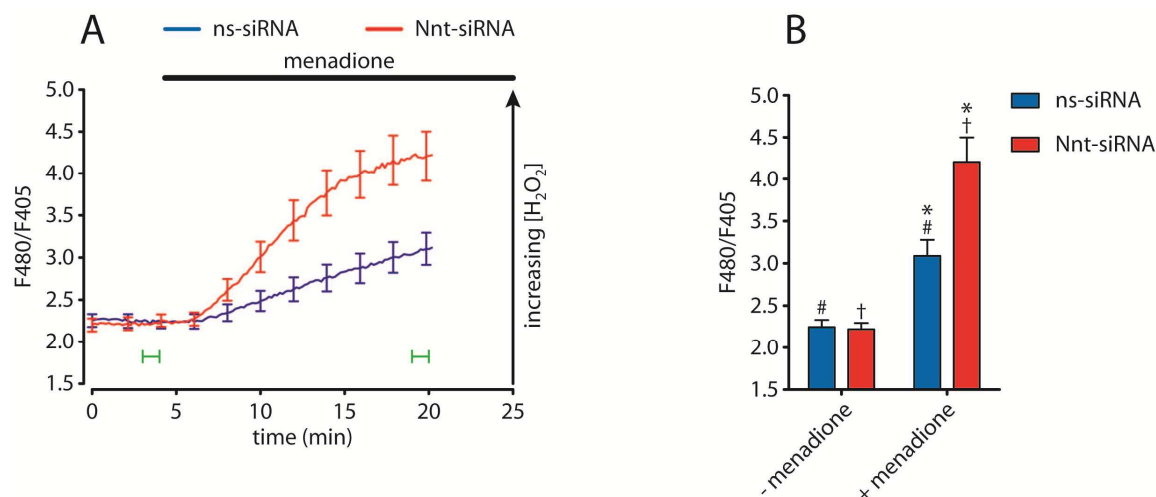


Fig. 3.9 Mitochondrial H₂O₂ dynamics in Nnt-silenced MIN6 cells upon menadione stimulation

MIN6 cells expressing HyPer were transfected with 50 nM Nnt-siRNA or 50 nM ns-siRNA. **A** Average HyPer fluorescence ratio from 23 ns-siRNA and 25 Nnt-siRNA transfected cells. SEM is shown for data points at 2 min intervals. **B** Mean data $\pm$ SEM in the absence or presence of menadione. Approximately 25 data points, corresponding to a time interval of 1 min (as indicated with green scale in A), were averaged. Data were collected from 3 dishes (ns-siRNA) and 4 dishes (Nnt-siRNA). Significance was tested using a two-way ANOVA with repeated measures and the Bonferroni post-test. Compared groups: ns-siRNA – menadione vs. ns-siRNA + menadione (#), Nnt-siRNA – menadione vs. Nnt-siRNA + menadione (†) and ns-siRNA + menadione vs. Nnt-siRNA + menadione (*) are significantly different at #, †, * $p < 0.001$.

3.3.5 H₂O₂ INCREASES INSIDE THE MITOCHONDRIA OF NNT DOWN-REGULATED MIN6 CELLS UPON GLUCOSE STIMULATION

Both ns-siRNA transfected and Nnt-silenced cells showed a stable H₂O₂ response throughout the entire recording when perfused with glucose-free solution (Fig. 3.10 A). This indicates that exposure to microscope light did not generate an increase in H₂O₂ directly (or increase in ROS and thereby H₂O₂ formation).

In ns-siRNA transfected cells 20 mM glucose produced a transient increase in HyPer fluorescence within 1 min of glucose application. The increase was followed by a return to basal levels after about 10 min and then by a slower secondary increase in H₂O₂ which plateaued after about 10 min (Fig. 3.10 B). Nnt-silenced cells displayed a response that had a similar time course. However, the increase in mitochondrial H₂O₂ on glucose stimulation was substantially larger.

Steady state fluorescence ratios exhibited a significant increase in both ns-siRNA transfected ($P < 0.05$) and Nnt-silenced ($P < 0.001$) cells upon glucose stimulation (Fig. 3.10 C). However, comparison of mitochondrial H₂O₂ under glucose stimulation between ns-siRNA transfected and Nnt-silenced MIN6 cells demonstrated a significantly larger increase ($P = 0.003$) in Nnt-silenced cells compared with ns-siRNA transfected (Fig. 3.10 C).

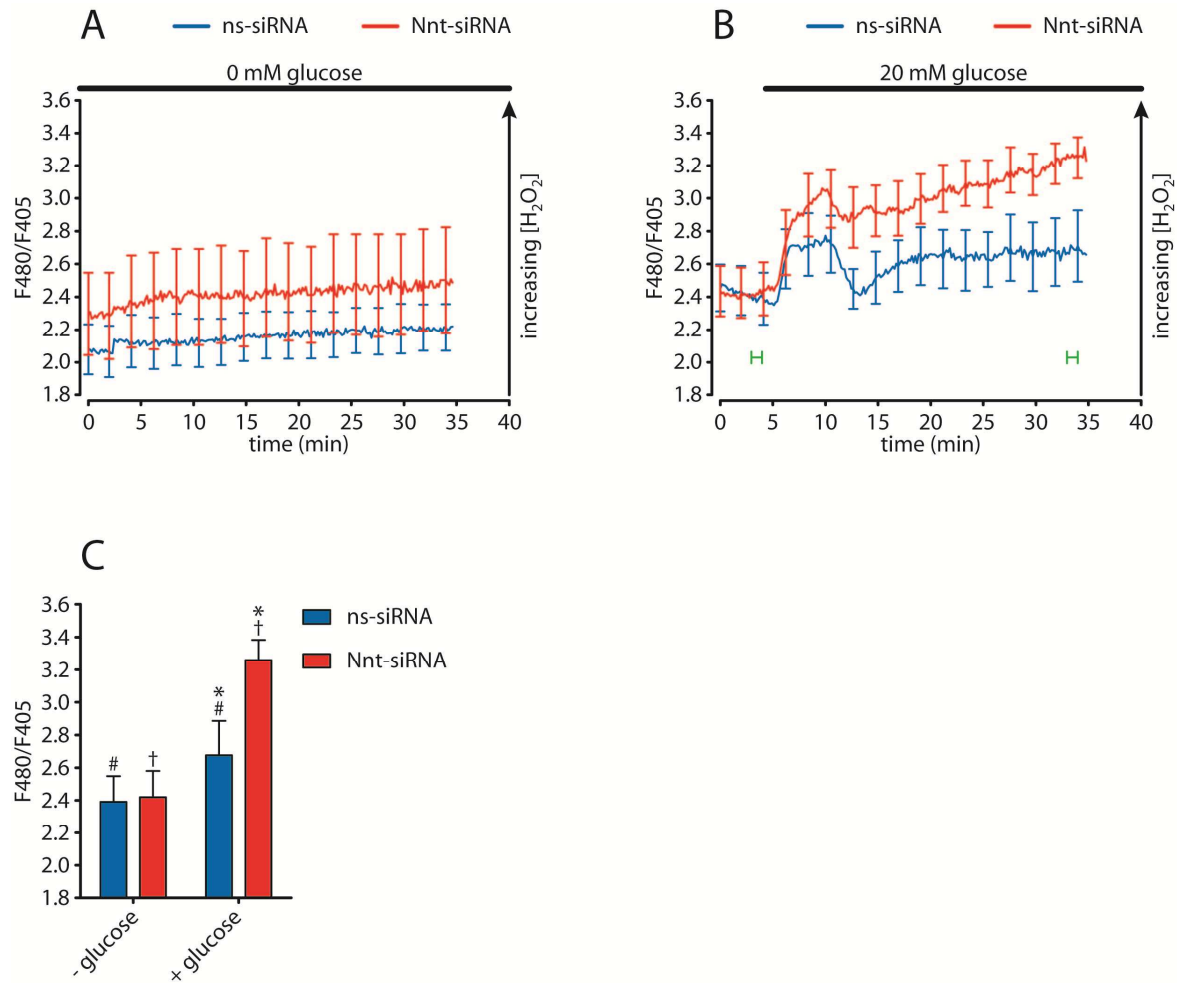


Fig. 3.10 Increase of mitochondrial H_2O_2 in Nnt-silenced MIN6 cells upon stimulation with 20 mM glucose

Min6 cells expressing HyPer were transfected with 50 nM Nnt-siRNA or 50 nM ns-siRNA. **A** Average HyPer fluorescence ratio from 5 ns-siRNA and 5 Nnt-siRNA transfected cells exposed to 0 mM glucose. SEM is shown for data points at 2 min intervals. **B** Average HyPer fluorescence ratio from 17 ns-siRNA and 14 Nnt-siRNA transfected cells exposed to 20 mM glucose. SEM is shown for data points at 2 min intervals. **C** Mean data $\pm$ SEM of ns-siRNA and Nnt-siRNA transfected cells in the absence or presence of glucose. Approximately 25 data points, corresponding to a time interval of 1 min (as indicated with green scale in B), were averaged. Data were collected from 5 dishes (ns-siRNA) and 5 dishes (Nnt-siRNA). Significance was tested using a two-way ANOVA with repeated measures and the Bonferroni post-test. Compared groups: ns-siRNA – glucose vs. ns-siRNA + glucose (#), Nnt-siRNA – glucose vs. Nnt-siRNA + glucose (†) and ns-siRNA + glucose vs. Nnt-siRNA + glucose (*) are significantly different at # $p < 0.05$, † $p < 0.001$, * $p < 0.05$.

3.3.6 EFFECT OF 1 mM GSH INCUBATION ON GLUCOSE-STIMULATED INCREASE IN $[Ca^{2+}]_i$ IN WILD-TYPE AND NNT MUTANT ISLETS

GSH is one of the most important antioxidants and cofactors that detoxify ROS in the mitochondria (Mari, Morales et al. 2009). It was previously shown that knockout of Nnt in *C. elegans* led to a strongly reduced GSH/GSSG ratio (Arkblad, Tuck et al. 2005). This suggests that when Nnt is non-functional the mitochondrial pool of GSH is depleted and is no longer sufficient to remove ROS from the β -cell, ultimately leading to a reduction in the influx of Ca^{2+} and insulin secretion.

Incubation of islets with GSH would therefore be predicted to increase the level inside the mitochondria, thus leading to an improved glucose-dependent Ca^{2+} influx and insulin secretion. In order to test this hypothesis I measured the effect of 10 mM glucose on $[Ca^{2+}]_i$ in Nnt mutant islets isolated from 1-year-old N68K and G745D mice. The islets were cultured for 24 hours in the presence of 1mM GSH. Islets were stimulated with 10 mM glucose, because this concentration had been previously found not to stimulate insulin secretion in both N68K and G745D islets (Freeman, Shimomura et al. 2006), whereas at 20 mM glucose there is a small but significant increase in insulin secretion in those islets (Cox 2007).

Wild-type (WT) islets maintained a stable, basal Ca^{2+} level at 2 mM glucose (Fig. 3.11 A). Stimulation with 10 mM glucose caused an initial decrease in $[Ca^{2+}]_i$ which was quickly followed by a robust rise in $[Ca^{2+}]_i$. After the Ca^{2+} peak, wild-type islets displayed an elevated and sustained level of Ca^{2+} which in most islets showed oscillations. Subsequent addition of a medium containing the K_{ATP} channel blocker tolbutamide and 2 mM glucose triggered a large and rapid increase in $[Ca^{2+}]_i$ (Fig. 3.11 E).

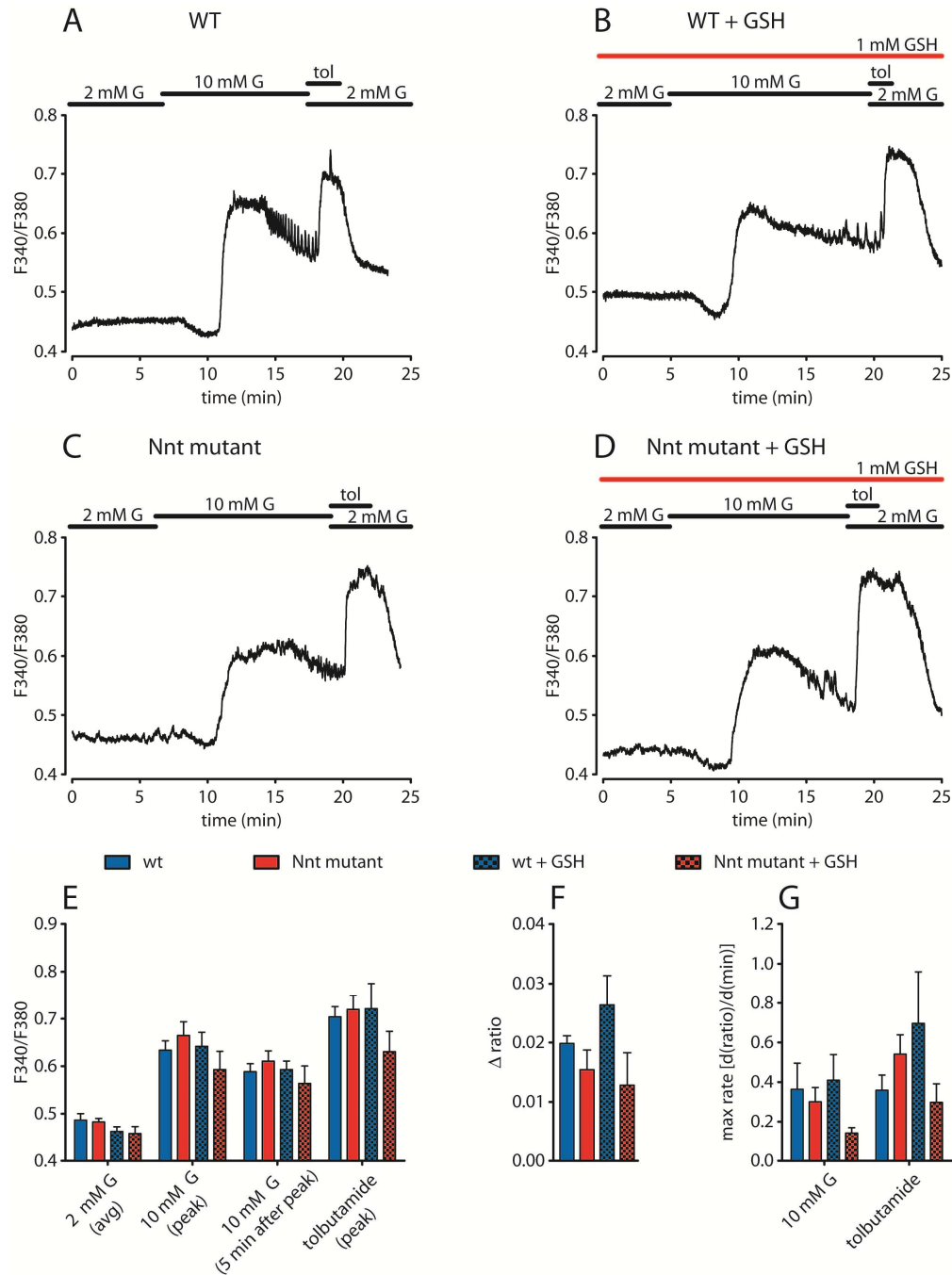


Fig. 3.11 Effect of 1 mM GSH on $[Ca^{2+}]_i$ in 1-year-old wild-type and Nnt mutant islets upon stimulation with 10 mM glucose and 0.2 mM tolbutamide plus 2 mM glucose

Islets were isolated from wild-type mice or N68K and G745D mutant mice (data were pooled and designated as Nnt mutant). **A-D** Representative Ca^{2+} traces from wild-type and Nnt mutant islets without GSH treatment (A, C) and with GSH treatment (B, D). **E** Mean data ($\pm$ SEM) of islets exposed to 2 mM glucose, 10 mM glucose (peak), 10 mM glucose (measurement taken 10 min after the peak) and 0.2 mM tolbutamide plus 2 mM glucose (peak). **F** Mean data ($\pm$ SEM), showing the nadir of initial fall in $[Ca^{2+}]_i$ on glucose stimulation, with $n = 8$ (wt), $n = 4$ (wt + GSH), $n = 4$ (Nnt mutant + GSH). **G** Mean data ($\pm$ SEM) for the maximum rate of change in $[Ca^{2+}]_i$ in response to glucose and tolbutamide. Data were obtained from a total of 4 wt mice and 4 Nnt mutant mice. Individual data set contains: 9 islets (wt – GSH); 5 islets (wt + GSH); 13 islets (Nnt mutant mice – GSH) and 5 islets (Nnt mutant mice + GSH). Significance levels were tested with a two-way-ANOVA test and no significance was indicated.

As expected, basal Ca^{2+} levels in 2 mM glucose solution were similar for wild-type and Nnt mutant islets. Moreover, there was no difference in the initial lowering of $[\text{Ca}^{2+}]_i$ on glucose stimulation (Fig. 3.11 F). Surprisingly, the glucose-stimulated $[\text{Ca}^{2+}]_i$ response of Nnt mutant islets was indistinguishable from wild-type islets (Fig. 3.11 C). As well as showing no difference in the mean increase in $[\text{Ca}^{2+}]_i$, Nnt mutant islets also exhibited oscillations in $[\text{Ca}^{2+}]_i$ on exposure to 10 mM glucose. Furthermore, the glucose-stimulated rise in $[\text{Ca}^{2+}]_i$ displayed comparable kinetics, when expressed as the maximum rate of change in $[\text{Ca}^{2+}]_i$ (Fig. 3.11 G). Tolbutamide, which directly shuts K_{ATP} channels and thereby bypassed the metabolic steps, produced a similar increase in $[\text{Ca}^{2+}]_i$ and the rate of the $[\text{Ca}^{2+}]_i$ response in islets of both genotypes (Fig. 3.11 E, G).

It has been shown previously that incubation with 1 mM GSH for 24 hours restored glucose-stimulated insulin in Nnt mutant islets from either N68K or G745D mice (personal communication Helen Freeman). However, the data presented here showed no effect of 1 mM GSH on wild-type or Nnt mutant islets on either glucose- or tolbutamide-stimulated $[\text{Ca}^{2+}]_i$ responses or on the basal $[\text{Ca}^{2+}]_i$ (Fig. 3.11 B, D, E). Furthermore, the maximum rate of increase of the $[\text{Ca}^{2+}]_i$ response was similar for all genotypes in both conditions, with and without GSH (Fig. 3.11 F).

This suggests that Nnt mutant islets are not different from wild-type islets in their $[\text{Ca}^{2+}]_i$ responses, in contrast to studies on MIN6 cells (Freeman, Shimomura et al. 2006) or isolated β -cells (Toye, Lippiat et al. 2005).

3.3.7 EFFECT OF 0.1 mM GSH INCUBATION ON GLUCOSE-STIMULATED INCREASE IN $[Ca^{2+}]_i$ IN C57BL/6J ISLETS

Since Nnt mutant islets did not display a reduced glucose-stimulated $[Ca^{2+}]_i$ response, I next used islets isolated from 8-9-month-old C57BL/6J mice. These C57BL/6J mice have a naturally occurring 5-exon deletion in their Nnt gene which results in a complete absence of the protein (Freeman, Hugill et al. 2006). These mice can therefore be considered a natural Nnt knockout mouse. I also tested the effect of GSH on glucose-stimulated $[Ca^{2+}]_i$ in C57BL/6J islets. The isolated islets were again incubated with GSH for 24 hours, but this time a concentration of 0.1 mM GSH was used because it was shown by Ammon et al. that 0.1 mM was the optimum concentration of GSH to augment glucose-stimulated insulin secretion in isolated rat islets (Ammon and Mark 1985).

Surprisingly, similar to Nnt mutant islets, C57BL/6J islets showed a robust increase in $[Ca^{2+}]_i$ upon stimulation with 20 mM glucose (Fig. 3.12). GSH had no effect on either basal $[Ca^{2+}]_i$ (measured at 2 mM glucose) or the initial fall of $[Ca^{2+}]_i$ produced by 20 mM glucose. Although both the initial and sustained $[Ca^{2+}]_i$ response produced by 20 mM glucose or by 0.2 mM tolbutamide plus 20 mM glucose were slightly enhanced when C57BL/6J islets were incubated in GSH, the difference was not significant (Fig. 3.12 C). Likewise, GSH-treated islets shared a slightly faster $[Ca^{2+}]_i$ response to the secretagogue, but this was not significant (Fig. 3.12 E).

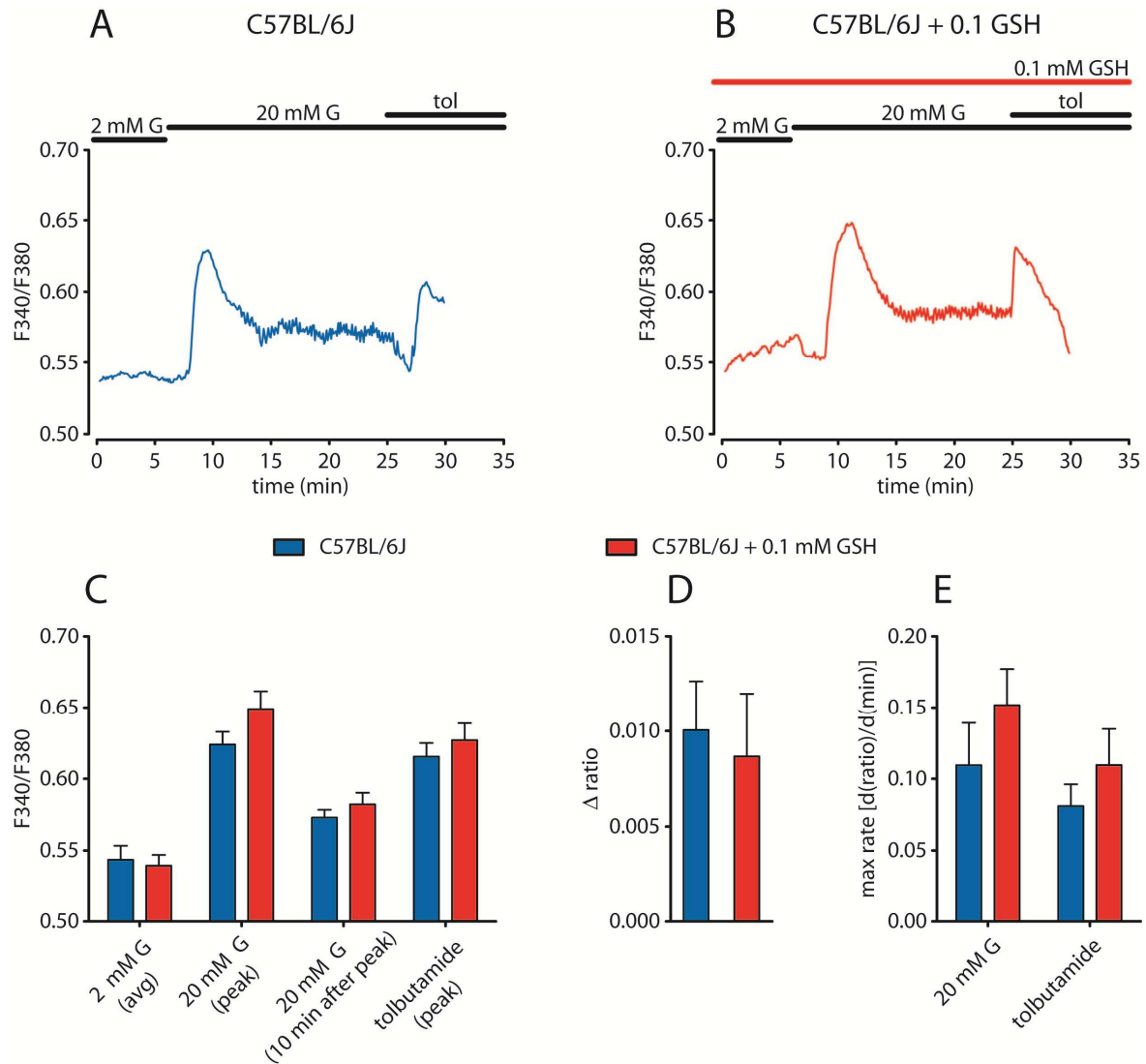


Fig. 3.12 Effect of 0.1 mM GSH on $[Ca^{2+}]_i$ in 8-9-month-old C57BL/6J islets upon stimulation with 20 mM glucose and 0.2 mM tolbutamide plus 20 mM glucose

A-B Representative Ca^{2+} traces from C57BL/6J islets without GSH treatment (A) and preincubated for 24 hours with 0.1 mM GSH (B). **C** Mean data ($\pm$ SEM) of islets exposed to 2 mM glucose (avg), 20 mM glucose (peak), 20 mM glucose (measurement taken 10 minutes after the peak) and 0.2 mM tolbutamide plus 20 mM glucose (peak), as indicated. **D** Mean data ($\pm$ SEM) for the nadir of initial fall in $[Ca^{2+}]_i$ on glucose stimulation, for C57BL/6J islets preincubated with (n = 11) or without (n = 12) GSH. **E** Mean data ($\pm$ SEM) for the maximum rate of change in $[Ca^{2+}]_i$ in response to glucose and tolbutamide. Data were obtained from 3 C57BL/6J mice (14 islets – GSH and 14 islets + GSH). Significance levels were tested with a non-paired Student's t-test and no significance was indicated.

3.3.8 COMPARISON OF GLUCOSE-STIMULATED $[Ca^{2+}]_i$ IN MALE 12-WEEK-OLD DBA/2 AND C57BL/6J ISLETS

Previous studies on insulin-secreting cells in which Nnt was ablated showed an absence of the glucose-stimulated $[Ca^{2+}]_i$ response (Toye, Lippiat et al. 2005; Freeman, Shimomura et al. 2006). This is in contrast to Nnt mutant or C57BL/6J islets which responded to glucose stimulation with an increase in $[Ca^{2+}]_i$ whose magnitude was similar to that in wild-type islets. In order to confirm that the differences in glucose-stimulated $[Ca^{2+}]_i$ response were not due to gender or age, I examined islets isolated from C57BL/6J mice which matched the gender and age of mice used in the published studies (Toye, Lippiat et al. 2005; Freeman, Shimomura et al. 2006). Islets from the DBA/2 strain, which expresses an increased level of the active form of Nnt (Aston-Mourney, Wong et al. 2007; Andrikopoulos 2010) were used as a positive control.

However, C57BL/6J islets still showed no difference in the $[Ca^{2+}]_i$ increase stimulated by 10 mM glucose even compared with DBA/2 islets (Fig. 3.13). The basal Ca^{2+} level in 2 mM glucose, the initial fall in $[Ca^{2+}]_i$ upon 10 mM glucose addition, the mean tolbutamide response and the maximum rates of change in $[Ca^{2+}]_i$ measured in response to glucose and tolbutamide were also not different (Fig. 3.13 C-E).

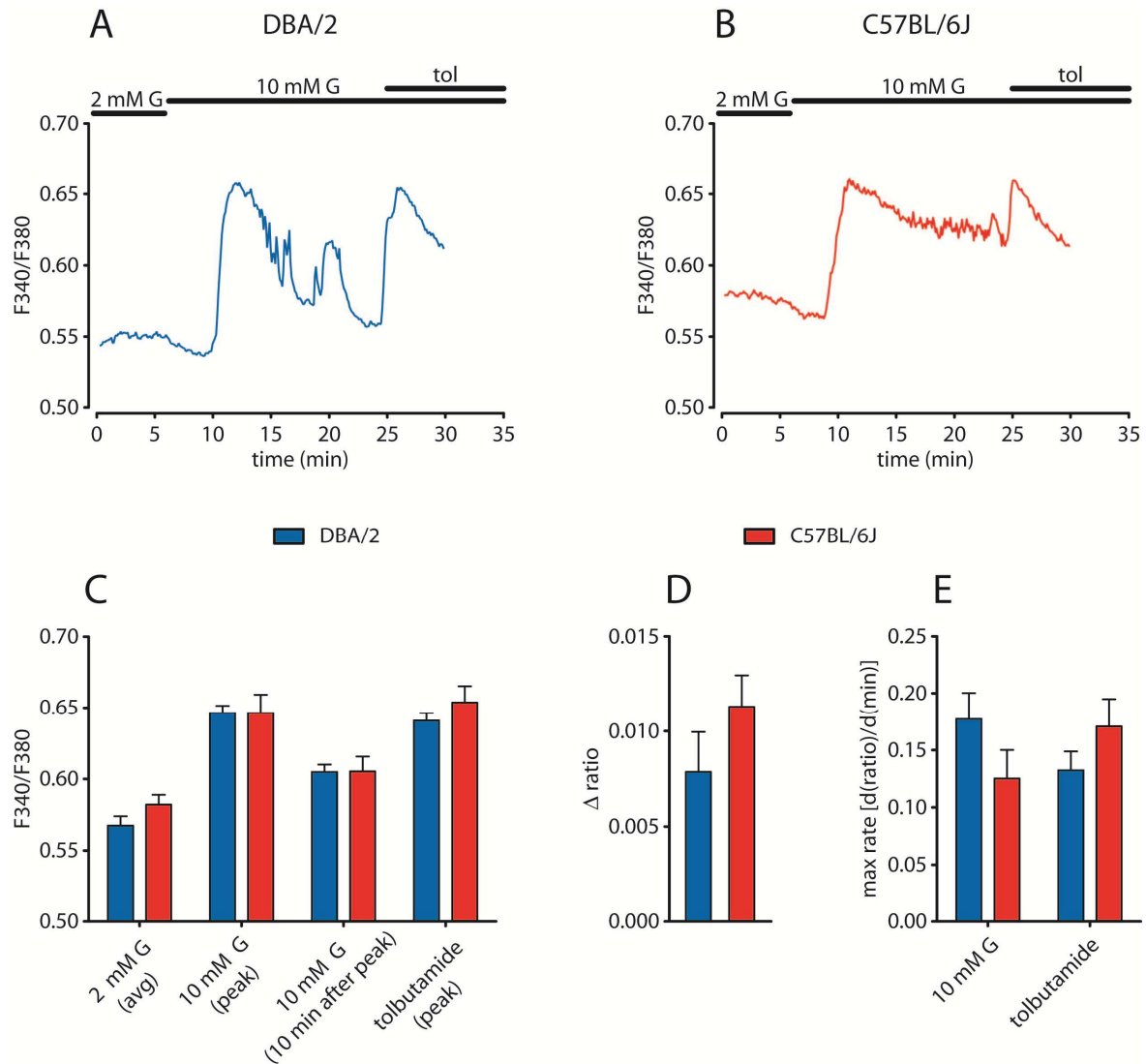


Fig. 3.13 Comparison of glucose- and tolbutamide-stimulated changes of $[Ca^{2+}]_i$ in islets isolated from 12-week-old male DBA/2 and C57BL/6J mice

A-B Representative Ca^{2+} traces from DBA/2 and C57BL/6J islets. **C** Mean data ($\pm$ SEM) for islets perfused with 2 mM glucose (steady-state), 10 mM glucose (peak), 10 mM glucose (measurement taken 10 minutes after the peak) and 0.2 mM tolbutamide plus 10 mM glucose (peak), as indicated. **D** Mean data ($\pm$ SEM), showing the nadir of initial fall in $[Ca^{2+}]_i$ on glucose stimulation for DBA/2 ($n = 8$) or C57BL/6J ($n = 11$). **E** Mean data ($\pm$ SEM) for the maximum rate of change in $[Ca^{2+}]_i$ in response to glucose and tolbutamide. Data were obtained from 3 DBA/2 mice (11 islets) and 3 C57BL/6J mice (11 islets). Significance levels were tested with a non-paired Student's t-test and no significance was indicated.

3.3.9 SUCCESSFUL GPx1 KNOCKDOWN WITH shRNA WAS VERIFIED BY (I) QUANTITATIVE PCR AND (II) WESTERN BLOT ANALYSIS

3.3.9.1 (I) QUANTITATIVE PCR

In order to investigate the functional role of GPx1 in β -cells, I used short hairpin RNA (shRNA) to silence the GPx1 gene in MIN6 cells. Transfected cells were positively selected by the puromycin (3 μ g/ml) antibiotic resistance conveyed by the shRNA vector. Control cells were transfected with a shRNA vector containing the puromycin resistance gene and a scrambled shRNA sequence with no homology to any known gene. Cells were assayed five days after transfection.

PCR efficiencies were used to determine the knockdown efficiency of the GPx1 gene (Fig. 3.14 A). See methods chapter for further details. The mean knockdown efficiency for cells transfected with GPx1 shRNA was approximately 70% (Fig. 3.14 A).

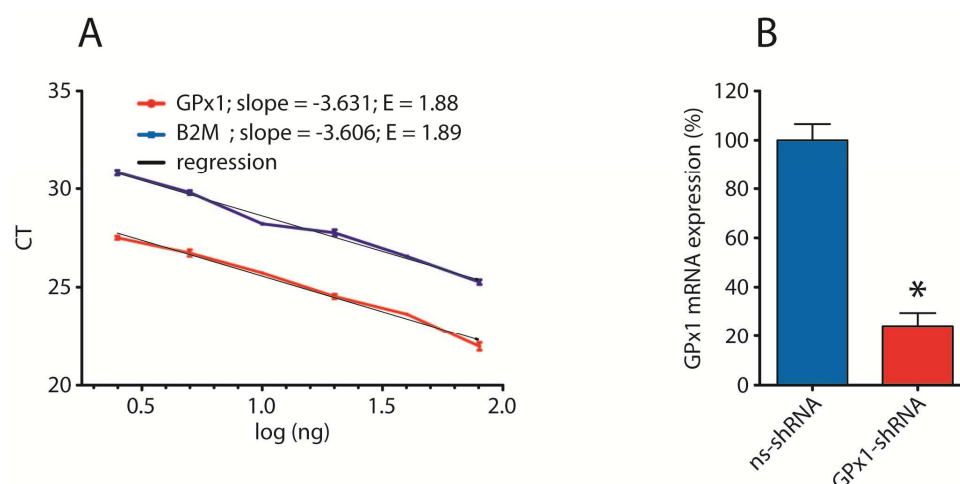


Fig. 3.14 Measurement of GPx1 gene expression using quantitative PCR

A Determination of real-time PCR efficiencies of the target gene (GPx1) and reference gene (B2M). Data points were mean $\pm$ SEM of 3 PCRs. See Chapter 2 (General methods) for further details. **B** Mean knockdown efficiency of GPx1 recorded from MIN6 cells transfected with nonsense shRNA (ns-shRNA) and GPx1-shRNA. Data are the mean $\pm$ SEM of 3 independent experiments. Significance was tested using an unpaired t-test. * $p < 0.05$.

3.3.9.2 (II) WESTERN BLOT ANALYSIS

Western blotting with antibodies to GPx1 and β -tubulin revealed a single band at ~ 22 kDa and ~ 55 kDa, respectively, in ns-shRNA transfected MIN6 cells (Fig. 3.15 A). Silencing of the GPx1 gene selectively reduced GPx1 protein expression without affecting the expression of β -tubulin. Quantification of GPx1 expression in GPx1-shRNA treated cells showed a knockdown of about 70% in comparison to ns-shRNA transfected cells (Fig. 3.15 A), consistent with the mRNA data.

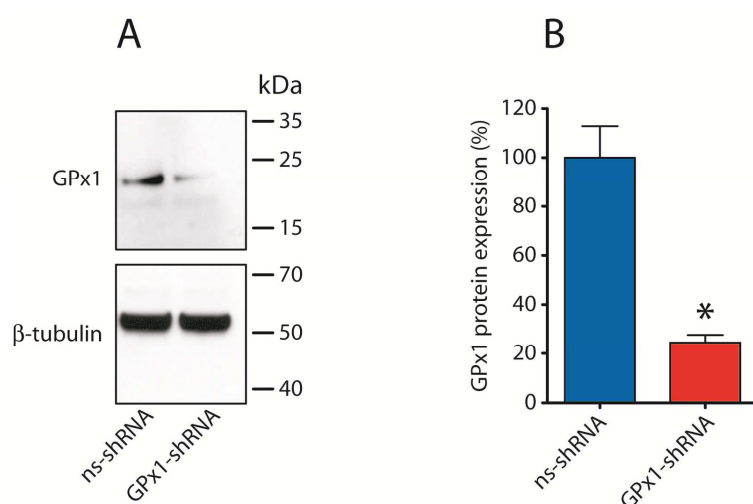


Fig. 3.15 Western blot analysis of ns-shRNA and GPx1-shRNA transfected MIN6 cells

A Each lane was probed with antibodies against GPx1 and β -tubulin, as indicated. **B** Quantitative analysis of Western blots. Data are mean $\pm$ SEM of 3 experiments. Significance was tested using an unpaired t-test. * $p < 0.05$.

3.3.10 INSULIN SECRETION IN GPx1-SILENCED MIN6 CELLS

I next investigated the effect of GPx1 on insulin release by comparing insulin secretion from GPx1-silenced MIN6 cells and ns-shRNA transfected cells. Figure 3.16 shows that basal insulin secretion measured at 2 mM glucose, as well as secretion in the response to a stimulatory concentration of glucose (20 mM) was significantly enhanced in GPx1 knockdown cells. Tolbutamide, in the presence of 2 mM glucose, also boosted insulin secretion to a significantly greater extent in GPx1

knockdown cells. This indicates that the enhanced insulin secretion was independent of glucose metabolism. Insulin content did not differ between ns-shRNA transfected and GPx1 knockdown cells, suggesting that there was no effect of GPx1 knockdown on the biogenesis of insulin (Fig. 3.16).

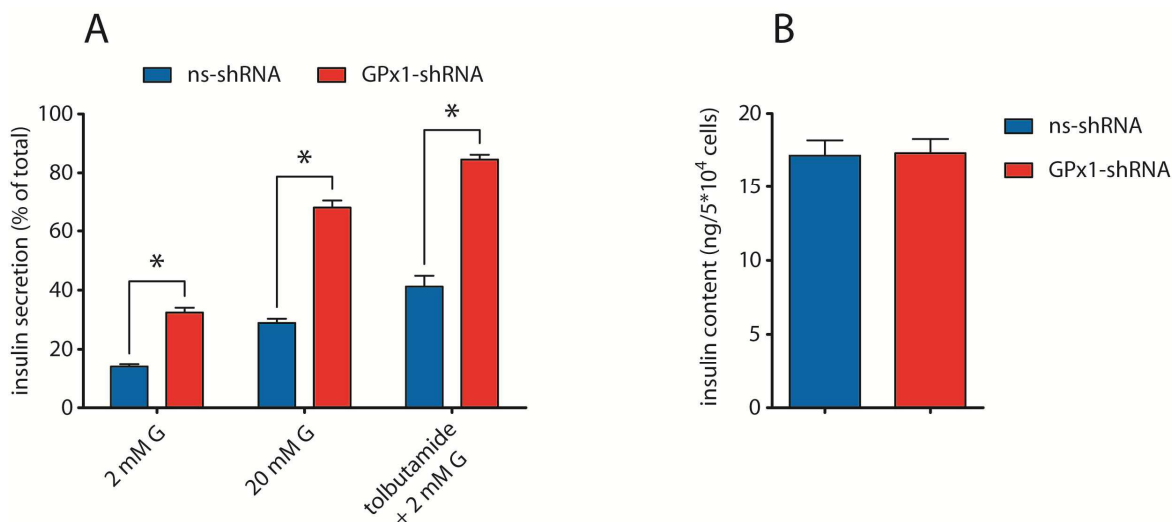


Fig. 3.16 Insulin secretion from GPx1-silenced MIN6 cells

A Insulin secretion in response to 2 mM glucose, 20 mM glucose and 0.2 mM tolbutamide plus 2 mM glucose in ns-shRNA and GPx1-shRNA transfected cells. Data are expressed as a percentage of insulin content. **B** Insulin content of ns-shRNA and GPx1-shRNA transfected cells. Bars express the mean $\pm$ SEM of 4 wells and are representative of three separate experiments. Significance was tested using an unpaired t-test. * $p < 0.05$.

3.3.11 MEASUREMENT OF $[Ca^{2+}]_i$ IN GPX1-SILENCED MIN6 CELLS

To elucidate the molecular mechanism of the enhanced insulin secretion, I measured changes in $[Ca^{2+}]_i$ upon glucose stimulation (20 mM) in ns-shRNA transfected and GPx1-silenced cells (Fig. 3.17). Imaged cells displayed a great heterogeneity in their response to glucose. This is characteristic of the MIN6 β -cell line and varies with the passage number and aggregation of cells (Hauge-Evans, Squires et al. 1999; Lilla, Webb et al. 2003). For the measurement of $[Ca^{2+}]_i$, only cells from passage number 26 – 30 were used and only cells in clusters of 2–3 were imaged.

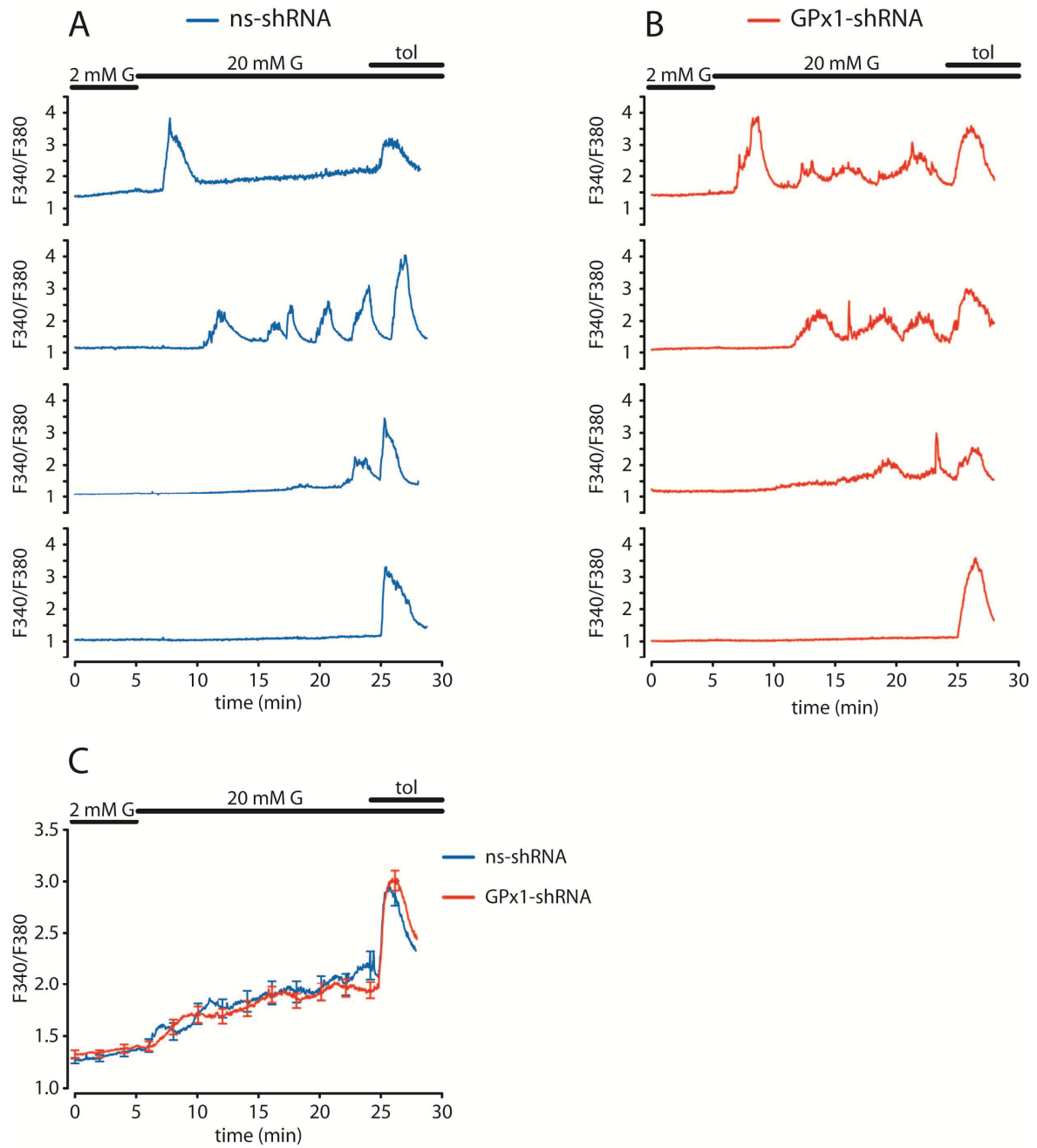


Fig. 3.17 Effect of GPx1 knockdown on $[Ca^{2+}]_i$ in MIN6 cells upon stimulation with glucose and tolbutamide

A, B Representative Ca^{2+} traces from ns-shRNA (A) and GPx1-shRNA (B) transfected cells stimulated with 20 mM glucose and 0.2 mM tolbutamide plus 20 mM glucose. **C** Average change in $[Ca^{2+}]_i$ from 45 ns-shRNA and 54 GPx1-shRNA transfected cells in response to glucose and tolbutamide. SEM is shown for data points at 2 min intervals. Data were collected from two separate experiments.

In ns-shRNA transfected cells 20 mM glucose elicited a rise in $[Ca^{2+}]_i$ in about 80% of the cells.

Representative traces are shown in figure 3.17 A. The glucose-induced changes in $[Ca^{2+}]_i$ were

heterogeneous: whereas some cells responded after 2 – 3 min, others showed a lag time of about 15 min. In most cells the rise in $[Ca^{2+}]_i$ was followed by Ca^{2+} oscillations. Only cells responding to tolbutamide, which depolarises the cells and causes a robust Ca^{2+} influx, were included in the analysis.

The pattern of glucose-induced changes in $[Ca^{2+}]_i$ were similar for GPx1-silenced cells and ns-shRNA transfected cells, with a mixture of early and late responses in about 80% of recorded cells (Fig. 3. 14 B). The average Ca^{2+} trace of GPx1-silenced cells was comparable to that of ns-shRNA transfected cells (Fig. 3.17 C). There was no difference in either basal $[Ca^{2+}]_i$ at 2 mM glucose or the mean amplitude of the $[Ca^{2+}]_i$ response upon 20 mM glucose stimulation. Tolbutamide evoked an equal response in both cell types.

3.3.12 H_2O_2 INCREASE INSIDE THE MITOCHONDRIA OF GPX1 DOWN-REGULATED MIN6 CELLS UPON GLUCOSE STIMULATION

GPx1 is located in the mitochondria and is considered to be the major GPx isoform that detoxifies H_2O_2 (Esworthy, Ho et al. 1997; Lee, Song et al. 2008; Mari, Morales et al. 2009). It is therefore expected that knockdown of GPx1 in β -cells will lead to an accumulation of mitochondrial H_2O_2 during glucose stimulation of glucose, similar to that found by silencing Nnt. To test this hypothesis, I measured glucose-induced (20 mM) mitochondrial H_2O_2 in GPx1-silenced MIN6 cells using the H_2O_2 fluorescent sensor HyPer (Fig. 3.18).

Following a 1 hour pre-incubation in glucose-free buffer, both cells types displayed a stable H_2O_2 trace in glucose free solution (Fig. 3.18 A, B). Upon stimulation with 20 mM glucose, H_2O_2 increased, but surprisingly the magnitude and the time course were not different between ns-shRNA transfected and GPx1 knockdown cells (Fig. 3.18 C).

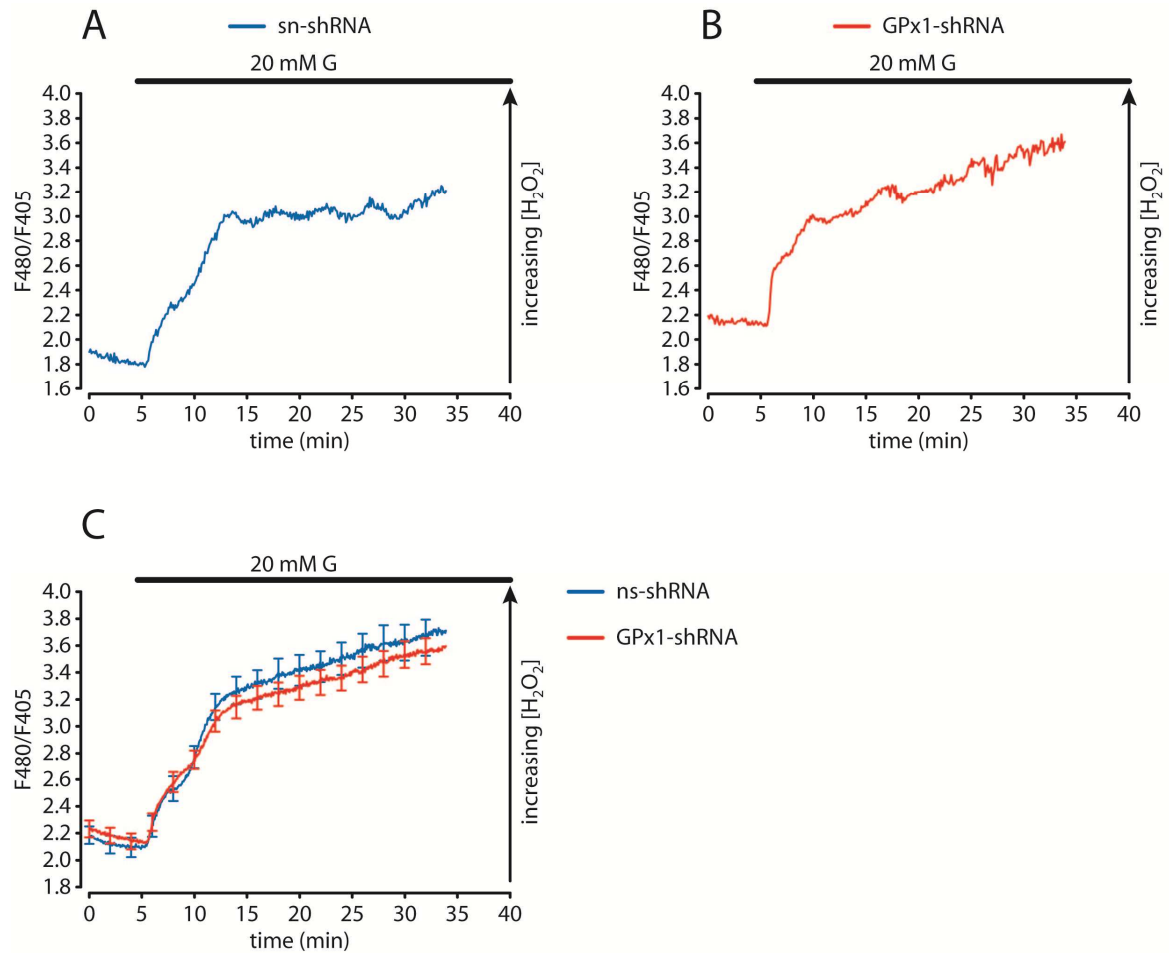


Fig. 3.18 Changes in mitochondrial H_2O_2 in GPx1-silenced MIN6 cells upon stimulation with glucose (20 mM)

A, B Representative traces of HyPer fluorescence ratio showing ns-shRNA (A) and GPx1-shRNA (B) transfected cells in response to 20 mM glucose. **C** Average HyPer fluorescence ratio from 76 ns-shRNA and 73 GPx1-shRNA transfected cells exposed to 20 mM glucose. SEM is shown for data points at 2 min intervals. Data were collected from four separate experiments.

3.4 DISCUSSION

3.4.1 GENE-SILENCING IN MIN6 CELLS USING siRNA OR shRNA

Knocking down the *Nnt* gene using siRNA showed a low reproducibility, with most of the assays producing inadequate knockdown (60% or less). The highest knockdown efficiency recorded was about 75%. The low reproducibility and average knockdown efficiency were independent of the sequence of the siRNA directed against *Nnt* (more than four different siRNAs were tested), the concentration of siRNA (5 – 100 nM) or the time of incubation (1 - 3 days). Since successful RNAi experiments depend on effective delivery of siRNA into cells, transfection efficiency was monitored by fluorescently labelled siRNAs. This indicated about 95% of cells were transfected. Furthermore, discrete fluorescent spots, probably corresponding to processing bodies (P bodies) associated with siRNA-induced mRNA degradation (Anderson 2005) were observed inside the cells suggesting that siRNAs were taken up by the cell. Thus, it is likely that the 60% measured for the whole population reflects the knockdown at the level of an individual cell. The 60% knockdown success of *Nnt* compares to nearly 100% efficiency reported by Freeman et al. (Freeman, Shimomura et al. 2006). However, other studies using siRNA gene silencing in MIN6 cells showed that knockdown efficiencies of 50 – 60% for a number of different genes were associated with a physiological effect (Iguchi, Ikeda et al. 2005; Doi, Shono et al. 2006).

No significant reduction of the *GPx1* gene was achieved using siRNA (purchased from different companies) and employing a range of incubations times and siRNA concentrations. I therefore used shRNA to silence the *GPx1* gene. Selection of the transfected cell via the antibiotic resistance did not seem to be a crucial factor for the overall knockdown success, since only two out of five shRNAs produced a knockdown of 70%.

3.4.2 NNT: ITS ROLE IN B-CELL METABOLISM

It was shown that MIN6 cells lacking Nnt generate significantly more H_2O_2 than wild-type cells when stimulated by menadione or glucose. Changes in H_2O_2 are robust in menadione-treated cells, which is not surprising considering that menadione, a powerful free radical generator, may generate H_2O_2 by at least two mechanisms (Halliwell and M.C. Gutteridge 2007):

- 1) as a quinone, menadione can participate in redox cycling, forming a semiquinone radical that in turn leads to an increase in $\text{O}_2^{\bullet-}$. Superoxide is then dismutated by SOD2 to O_2 and H_2O_2 (Fridovich 1995).
- 2) Menadione could react with GSH to deplete the pool of reduced glutathione and impair the ability of glutathione peroxidases to remove H_2O_2 (Shi, Iwamoto et al. 1994).

I used two methods of detecting ROS, both of which have different advantages and disadvantages. Reduced dichlorodihydrofluorescein (H_2DCF) can easily be loaded into the cell where it is oxidized by some reactive oxygen species, leading to a fluorescent signal. Indeed, H_2DCF was effective in detecting ROS generation by menadione. However, H_2DCF failed to detect any change in ROS in response to glucose stimulation. Furthermore, H_2DCF oxidation is not specific for any particular form of ROS (Myhre, Andersen et al. 2003) and has been reported to be photo-oxidized (Bilski, Belanger et al. 2002).

The fluorescent protein HyPer enables mitochondrial H_2O_2 dynamics to be measured under physiological conditions. However, HyPer is not without its disadvantages. As reported for most sensors based on YFP, HyPer fluorescence depends on pH (Belousov, Fradkov et al. 2006). The titration curve of HyPer shows that with increasing pH, similar to an increase of ROS, the fluorescence ratio of 480 nm and 405 nm increases concomitantly. Thus changes in HyPer fluorescence may reflect changes in pH rather than ROS. Glucose-dependent alkalization of the mitochondrial matrix has been observed in insulin-secreting cells (Wiederkehr, Park et al. 2009;

Akhmedov, Braun et al. 2010) and thus the observed increase in HyPer fluorescence may be due to this. However, alkalization following nutrient stimulation (including glucose) plateaus after approximately 10 min and is maintained (Wiederkehr, Park et al. 2009). In contrast, exposure of either menadione or glucose to MIN6 cells still increased HyPer fluorescence after 10 min. This suggests that the HyPer signal observed after 10 min is due to an increase in ROS.

Pancreatic islets isolated from C57BL/6J mice responded to glucose stimulation with a robust increase in $[Ca^{2+}]_i$. The initial rise as well as the secondary increase of $[Ca^{2+}]_i$ in these islets was comparable to DBA/2 islets, which express functional Nnt. This finding was unexpected, given that previous studies of C57BL/6J β -cells (Toye, Lippiat et al. 2005) and MIN6 cells in which Nnt was silenced with siRNA, (Freeman, Shimomura et al. 2006) only displayed a marginal increase in $[Ca^{2+}]_i$ following glucose stimulation. However, an important difference in these studies is that one was carried out on islet cells and the other on a cell line. Dispersing the islet into single cells has the disadvantage that it disrupts the natural environment for β -cells, in which intercellular signalling can occur via both chemical (paracrine) and electrical signals (Rorsman, Berggren et al. 1989; Charollais, Gjinovci et al. 2000), resulting in greater heterogeneity in response to glucose and much poorer insulin secretion from isolated β -cells than in whole islets (Herchuelz, Pochet et al. 1991; Luther, Hauge-Evans et al. 2006). Importantly, single β -cells and MIN6 cells, in contrast to β -cells within the intact islet, are directly exposed to a hyperoxic ROS-creating environment in cell culture (Halliwell 2003). This state of oxidative stress on Nnt-ablated cells may make a critical contribution to the reduction in ATP synthesis and the failure to close ATP channels and trigger insulin release. This is illustrated by the observation that Nnt ablation had no effect on growth in *C. elegans* under normal conditions. However, under conditions of oxidative stress the growth of worms lacking Nnt activity was severely impaired (Arkblad, Tuck et al. 2005).

Measurements of glucose-stimulated $[Ca^{2+}]_i$ in Nnt mutant islets isolated from both N68K or G745D mice also revealed a robust increase in $[Ca^{2+}]_i$ and no difference in the magnitude or

time course of the response to wild-type islets. This was initially surprising because of the compelling evidence provided by Freeman et al. for a proposed Ca^{2+} -dependent mechanism of Nnt-impaired insulin secretion (Freeman, Shimomura et al. 2006). However, in parallel experiments, other lab members showed that there was also no difference in insulin secretion between wild-type mice and either N68K or G745D mice, *in vitro* as well as *in vivo*. In glucose tolerance tests it appeared that the wild-type mice were now as glucose intolerant as the mutant mice. Further investigations revealed that in the process of transferring these mice into a new animal house they had been backcrossed onto a different C3H background (Prof Roger Cox, personal communication). This mouse had a genetically different background as it had larger ears. Thus, it seems likely that other, modifying genes were therefore obscuring the effect of the Nnt deletion. This idea was supported by the original study of Toye et al. which revealed that the genetic locus harbouring the Nnt gene only explained about 10% of the total variance in glucose intolerance of the C57BL/6J mouse (Toye, Lippiat et al. 2005).

The strain dependency of Nnt on the resultant phenotype has been underscored by recent studies. The research group of Sofianos Andrikopoulos observed that congenic C57BL/6J mice with full-length Nnt from the DBA/2 strain showed comparable insulin secretion to wild-type C57BL/6J mice with a five-exon deletion (Aston-Mourney, Wong et al. 2007). Further studies comparing the substrain C57BL/6J with C57BL/6N, that differs from C57BL/6J in carrying the wild-type Nnt gene, revealed no difference in physiological parameters including insulin secretion and glucose tolerance (Wong, Blair et al. 2010). These findings contrast with the observation that ablation or mutation of Nnt in a non-C57BL/6J background (C3H/HeH and a mixed 129/Sv and C57BL/6 strain) results in mice that are glucose intolerant and have decreased plasma insulin or insulin secretion (Freeman, Shimomura et al. 2006; Parker, Vidal-Puig et al. 2009).

The same dependence on genetic background was also observed for the UCP2 gene: on a mixed 129/Sv×C57BL/6 background $\text{Ucp2}^{-/-}$ mice showed dramatically enhanced glucose-

stimulated insulin secretion (Zhang, Baffy et al. 2001), whereas on a pure C57BL/6J, A/J, or 129/SvImJ background *Ucp2*^{-/-} mice displayed diminished glucose-stimulated insulin secretion (Pi, Bai et al. 2009).

Interestingly C57BL/6J mice, expressing a non-functional *Nnt* at comparable levels to the wild-type *Nnt* of C57BL/6N, also exhibited equal *Nnt* enzyme activity as measured by the conversion of NADP⁺ to NADPH. This suggests that other enzymes are able to compensate for the functional loss of *Nnt* in reducing NADP⁺ to NADPH (Aston-Mourney, Wong et al. 2007). It is estimated that 45% of cellular NADPH is provided by the action of *Nnt*. Further sources of NADPH include the pentose phosphate pathway in the cytosol and both NADP-isocitrate dehydrogenase and decarboxylating malate dehydrogenase (malic enzyme) in the mitochondria (Rydstrom 2006; Sheeran, Rydstrom et al. 2010). Indeed, Vogel et al. demonstrated that in mitochondria isolated from rat forebrain all three enzymes (*Nnt*, NADP-isocitrate dehydrogenase and malic enzyme) contribute to the formation of NADPH which, in turn, was linked to the regeneration of GSH (Vogel, Wiesinger et al. 1999). It is therefore conceivable that the expression or activity of either NADP-isocitrate dehydrogenase or malic enzyme is upregulated in C57BL/6J mice in response to a chronic non-functional *Nnt*. The time needed for these compensatory mechanisms to develop may explain why transient manipulation of *Nnt* expression produces phenotypical changes; knockdown of *Nnt* in MIN6 cells led to an increase in mitochondrial H₂O₂, as demonstrated in this thesis (Shimomura, Galvanovskis et al. 2009) and reduced glucose-stimulated insulin secretion mediated by a reduction in [Ca²⁺]_i (Freeman, Shimomura et al. 2006). Conversely, overexpression of *Nnt* in MIN6 cell resulted in a 2.5-fold increase in glucose-stimulated insulin secretion (Wong, Blair et al. 2010). In the Wong paper it was suggested that it was the increase in *Nnt* expression, as seen in the DBA/2 strain (Aston-Mourney, Wong et al. 2007) that is responsible for enhancing insulin secretion. Furthermore, they argued that introducing a functional *Nnt* gene into the C57BL/6J genome by the means of a bacterial artificial chromosome, as it was done in the study of Freeman et al. (Freeman, Hugill et al. 2006), causes an overexpression of *Nnt*. Consequently it

was this overexpression that rescued the impaired insulin secretion and glucose-intolerant phenotype of the C57BL/6J mouse.

Compensatory mechanism induced by loss of *Nnt* function may explain why, to date, there have been no mutations found in the *Nnt* gene in human type 2 diabetic patients. Moreover, it was reported by Aston-Mourney et al. that no genome-wide study for type 2 diabetes susceptibility had shown significant linkage to the chromosome region containing *Nnt* (Aston-Mourney, Wong et al. 2007).

Previous studies by our group have shown that overnight culture of *Nnt*-mutant islets in 1mM GSH is able to partly restore glucose-stimulated insulin release (Helen Freeman personal communication). This study, however, failed to show any significant increase in glucose-stimulated $[Ca^{2+}]_i$ in non-functional *Nnt* islets following GSH incubation. This suggests that GSH is likely to act downstream of increase in $[Ca^{2+}]_i$ to enhance insulin secretion.

GSH is synthesized in the cytoplasm of all animal cells. Apart from being a crucial cofactor for antioxidant enzymes that remove ROS, GSH can also act as an antioxidant. Mitochondria contain about 10-20% of total cellular GSH but must acquire it from the cytoplasm (Fernandez-Checa and Kaplowitz 2005). GSH can cross the outer mitochondrial membrane through porin channels and is transported into the matrix by at least two antiport carrier proteins (Mari, Morales et al. 2009). Incubating MIN6 cells with 1 mM GSH increases its intracellular concentration (Helen Freeman, personal communication). Assuming that GSH can be taken up by the islets, it is likely that once inside the β -cell GSH can enter the mitochondria and be utilized as an electron donor by peroxidases to remove ROS or repair oxidative damage. This idea is supported by the findings of Bertram et al. who showed that GSH incubation of retinal pigment epithelial cells was able to attenuate the loss of mitochondrial membrane potential caused by cigarette-smoke-induced oxidative stress (Bertram, Baglolle et al. 2009).

Reports of the effect of exogenous GSH on insulin secretion are ambiguous. Whereas Pi et al. described a decrease in insulin release at both non-stimulatory and stimulatory glucose concentrations in the insulin-secreting cell line INS-1 upon GSH incubation (Pi, Zhang et al. 2010), Ammon et al. demonstrated that exposure of GSH to pancreatic islets increased glucose-stimulated insulin secretion (Ammon and Mark 1985). In both studies similar concentrations (1 mM) and incubations times of GSH were used. In type 2 diabetic islets, GSH incubation (34 μ M) for 24 hours improved the secretory response to glucose significantly (Del Guerra, Lupi et al. 2005). These data are consistent with an *in vivo* study on elderly people with impaired glucose tolerance, which showed that GSH infusion potentiated glucose-stimulated insulin secretion but had no effect on control subjects with normal glucose tolerance (Paolisso, Giugliano et al. 1992). In this context it is noteworthy that on the new background both glucose tolerance and insulin secretion were not different between Nnt-mutant and wild-type mice (observation made by other members of the group) and that therefore GSH incubation was unlikely to increase glucose-stimulated $[Ca^{2+}]_i$ solely in Nnt mutant islets.

3.4.3 GLUTATHIONE PEROXIDASE 1 (GPx1): ITS ROLE IN INSULIN SECRETION

Silencing GPx1 in MIN6 cells resulted in hypersecretion of insulin which was independent of cytosolic Ca^{2+} and had no effect on mitochondrial ROS generation. It is surprising that GPx1 knockdown did not increase mitochondrial H_2O_2 production given the fact that GPx1 is considered to be a crucial enzyme in neutralizing H_2O_2 in the mitochondria (Panfili, Sandri et al. 1991; Esworthy, Ho et al. 1997; Legault, Carrier et al. 2000; Mari, Morales et al. 2009). However, it cannot be ruled out that the removal of H_2O_2 was mediated by enzymes other than GPx1. In mitochondria at least three other enzyme systems are known to catalyze the reduction of H_2O_2 to water:

- 1) a mitochondrial form of GPx4 (Conrad, Schneider et al. 2007).
- 2) mitochondrial forms of peroxiredoxins 1 and 5 (PRx1 and PRx5) which belong to the family of Se-independent peroxidases (Kalinina, Chernov et al. 2008). Peroxiredoxins use thioredoxin, another thiol-containing small protein, as an electron donor (Halliwell and M.C. Gutteridge 2007).
- 3) a possible role for glutaredoxin 2 was suggested by Fernando et al. (Fernando, Lechner et al. 2006).

Glutaredoxins are small thiol-containing oxidoreductases that use GSH as cofactor to reduce protein disulfides on target molecules (Fernandes and Holmgren 2004; Kalinina, Chernov et al. 2008). They are involved in multiple cellular processes such as DNA synthesis (Holmgren 1976), protein folding and transcriptional regulation (Lundstrom-Ljung and Holmgren 1995), as well as defence against oxidative stress and apoptosis (Daily, Vlamis-Gardikas et al. 2001). In the pancreatic β -cell a cytosolic form of glutaredoxin, glutaredoxin 1 (GRx1), was found to stimulate exocytosis of insulin (Ivarsson, Quintens et al. 2005; Reinbothe, Ivarsson et al. 2009). It was demonstrated that knockdown of GRx1 by RNA interference reduced glucose-stimulated insulin secretion in both insulin-secreting clonal cells and rat islets (Reinbothe, Ivarsson et al. 2009). Conversely, capacitance recordings on single cells showed a reduction in exocytosis in GRx1-silenced cells (Reinbothe, Ivarsson et al. 2009), whereas overexpression or addition of GRx1 to the pipette solution (Ivarsson, Quintens et al. 2005) increased exocytosis. Moreover, the two studies showed that glucose induced generation of NADPH. This pyridine nucleotide in turn enhances Ca^{2+} -induced exocytosis in pancreatic β cells. This effect is mediated by GRx1, since ablation of the protein abolishes NADPH-stimulated exocytosis. The authors of these papers suggest that electrons flow from NADPH (over glutathione reductase and then GSH) to GRx1. Consequently, GRx1 is able to reduce proteins relevant for the redox-dependent control of insulin exocytosis, resulting in an amplification of glucose-dependent insulin secretion.

GPx1 is expressed in the cytosol as well as in the mitochondria. Therefore, it is possible that silencing of GPx1 would lead to an increase in the ratio of GHS to GSSG in the cytosol. The increased level of reduced glutathione could be used by GRx1 to potentiate Ca^{2+} -dependent exocytosis. Interestingly, intracellular addition of glutathione applied through the pipette solution was shown to stimulate exocytosis in mouse β -cells (Ivarsson, Quintens et al. 2005). This would provide a mechanism by which ablation of GPx1 enhances the activity or expression level of GRx1, leading to an increase in insulin secretion.

Alternatively, GPx1 knockdown could lead to an increase in the level of cytosolic H_2O_2 . This substance can engage in redox reactions that have an impact on intra and intercellular signalling (redox regulation). The principle of redox regulation is the reversal oxidation and reduction of SH groups on target proteins. Indeed, ROS generation is not always a harmful waste product: it can also fulfil essential functions in many biological processes. ROS plays a role in functional modification of, for example, ion transport, protein phosphorylation and dephosphorylation or redox-dependent transcriptional regulation (Halliwell and M.C. Gutteridge 2007; Pi, Zhang et al. 2010). The specific role of H_2O_2 in insulin secretion is discussed further below.

It is noticeable that the secondary increase in mitochondrial H_2O_2 was more pronounced in cells transfected with ns-shRNA than ns-siRNA (used as the control for knocking down Nnt). A possible explanation could be that the passage number of the cells was different. It has been known that MIN6 cells display functional heterogeneity with increasing passage number (Lilla, Webb et al. 2003) which could have an impact on the cell's handling of ROS. For example, Moran et al. showed a direct link between an increase in ROS and higher passage numbers of the osteosarcoma cell line U2OS (Moran, Shen et al. 2009). Another explanation could be the ns-shRNA control vector that expresses the antibiotic resistance gene puromycin n-acetyl transferase which was recently been found to cause aggregation of critical multifunctional

proteins which was associated with the development of intracellular ROS (Moran, Shen et al. 2009).

MIN6 cells in which the GPx1 gene was silenced displayed no differences in the magnitude and time course of their glucose-stimulated $[Ca^{2+}]_i$ to ns-shRNA transfected cells. Oxidative stress has been associated with dysregulated Ca^{2+} metabolism, generally causing a rise in $[Ca^{2+}]_i$. ROS can oxidize essential SH groups on ion channels transporters or pumps, interfering with, for example, the Ca^{2+} uptake into the ER (Dremina, Sharov et al. 2007) or Ca^{2+} efflux out of the cell. In particular the TRPM2 cation channel has been implicated in mediating an increase in $[Ca^{2+}]_i$ in pancreatic β -cells (Herson and Ashford 1999; Kuhn, Heiner et al. 2005). TRPM2 channels are non-selective and permeable for Ca^{2+} , Na^+ and K^+ , thus activation of TRPM2 in the plasma membrane may lead to depolarisation triggering a further increase of Ca^{2+} through voltage-gated Ca^{2+} channels. However, an alteration of Ca^{2+} metabolism does not seem to be involved in the physiological consequences of GPx1 knockdown since no change in $[Ca^{2+}]_i$ was observed.

Silencing GPx1 in MIN6 cells produced hypersecretion of insulin. Although a reduction in cell number during the knockdown of GPx1 was not observed it cannot be ruled out that GPx1-shRNA treated cells were damaged and consequently leaked insulin. This would explain the elevated secretion observed in 2 mM glucose. Cell viability tests are necessary to investigate this hypothesis. It is also possible that GPx1 knockdown stimulates insulin secretion independently of secretagogues downstream of Ca^{2+} influx. This process is likely to be modulated by H_2O_2 . This molecule has been implicated as a second messenger in a variety of physiological processes including proliferation, enhanced DNA repair and apoptosis (Rhee, Chang et al. 2003). H_2O_2 is a small uncharged molecule that can rapidly be synthesized and removed and is highly specific for thiol oxidation on certain proteins (D'Autreaux and Toledano 2007). There is now substantial evidence that ROS, in particular H_2O_2 , produced by glucose metabolism, act as metabolic signalling molecules for glucose-stimulated insulin secretion in pancreatic β -cells. Wollheim's

group observed that H_2O_2 and alloxan, a redox cycling toxin which was found to increase intracellular H_2O_2 , caused an increase in insulin release in rat islets at nonstimulatory glucose concentrations. This increase in insulin secretion was mediated by an increase in $[\text{Ca}^{2+}]_i$, which was attributed to inhibition of Ca^{2+} pumping by SERCA into the ER and the Ca^{2+} influx from the extracellular space. However, in the presence of stimulatory concentrations of glucose, $[\text{Ca}^{2+}]_i$ and insulin release were both reduced (Janjic, Maechler et al. 1999; Maechler, Jornot et al. 1999). Similarly, short term exposure to alloxan and xanthine oxidase/hypoxanthine, a well known $\text{O}_2^{\bullet-}$ generation system, led to an increase in insulin release at nonstimulatory glucose concentrations in rat islets (Ebelt, Peschke et al. 2000).

In other studies exogenous H_2O_2 , diethyl maleate (which enhances intracellular H_2O_2 levels) (Pi, Bai et al. 2007), as well as mitochondrial ROS induction (Leloup, Tourrel-Cuzin et al. 2009) increased insulin secretion in INS-1 cells or isolated mouse islets at non-stimulatory glucose concentrations. Interestingly, exogenous antioxidants inhibited the glucose-stimulated insulin secretion (Pi, Bai et al. 2007; Leloup, Tourrel-Cuzin et al. 2009) which was mediated by an decrease of $[\text{Ca}^{2+}]_i$ (Leloup, Tourrel-Cuzin et al. 2009). However, these authors also found that exogenous antioxidants treatments inhibit KCl-induced insulin release (Pi, Bai et al. 2007) which provides evidence that H_2O_2 acts downstream of Ca^{2+} influx to enhance insulin secretion. The role of ROS as a signalling molecule in glucose-stimulated insulin secretion further emphasises the action of NAD(P)H oxidase in production of $\text{O}_2^{\bullet-}$ in the cytosol in response to glucose, and inhibition caused a reduction in insulin secretion (Morgan, Rebelato et al. 2009). Altogether, these findings could explain how down-regulation of GPx1 in MIN6 cells could increase cytosolic H_2O_2 and in turn stimulate insulin secretion downstream of Ca^{2+} influx.

3.5 SUMMARY

Loss of Nnt function is linked a reduction in insulin secretion. It has been proposed that non-functional Nnt leads to ROS-mediated uncoupling of mitochondrial metabolism and consequently to a reduction in ATP and insulin secretion. The exact nature of ROS and the mechanism of its detoxification, however, have been unknown. In this study it was shown that ablation of Nnt in MIN6 cells resulted in a stronger increase of mitochondrial H_2O_2 upon stimulation with glucose or menadione than in ns-siRNA transfected cells. However, silencing of GPx1, a candidate gene for removing H_2O_2 from the mitochondria, had no effect on mitochondrial H_2O_2 and was linked to Ca^{2+} -independent hypersecretion of insulin, suggesting a stimulatory role of ROS in insulin secretion. Furthermore, exogenous GSH (both 0.1 mM and 1 mM) which was reported to stimulate insulin secretion, had no effect on glucose-stimulated $[\text{Ca}^{2+}]_i$ responses in either wild-type or Nnt mutant islets (islets isolated from either C57BL/6J, N68K or G745D mice). This suggests that GSH is likely to stimulate insulin secretion downstream of Ca^{2+} influx. In contrast to previous studies on Nnt mutant insulin-secreting cells (Toye, Lippiat et al. 2005; Freeman, Shimomura et al. 2006), I found that in Nnt mutant islets glucose-stimulated increase in $[\text{Ca}^{2+}]_i$ was not impaired but was similar to wild-type islets. This finding was associated with normal glucose tolerance and insulin secretion observed in N68K or G745D mutant mice and was probably caused by introducing the Nnt mutation onto a different background. The explanation of background dependency of the Nnt mutation is supported by recent studies (Aston-Mourney, Wong et al. 2007; Wong, Blair et al. 2010).

4 MOUSE MODELS OF NEONATAL DIABETES EXPRESSING A GAIN-OF-FUNCTION MUTATION (V59M) IN THE KIR6.2 SUBUNIT OF THE KATP CHANNEL

4.1 INTRODUCTION

4.1.1 NEONATAL DIABETES

Neonatal diabetes (ND) is a rare, monogenic form of diabetes that affects about 1 in 200,000 live births (Hattersley and Ashcroft 2005). The onset of diabetes occurs within the first six months of life, and it can be either permanent (PNDM) or transient (TNDM). Newborn infants with ND are characterised by low birth weight (mean 2,500 g). This is because the foetus does not secrete enough insulin, which is a major foetal growth factor in the third trimester of pregnancy (Murphy, Ellard et al. 2008). About 20% of patients with ND also present neurological complications. The relative rare, but severe, DEND syndrome consists of *developmental delay*, *epilepsy* and *neonatal diabetes*. The more commonly occurring syndrome, *intermediate DEND* (iDEND), is characterised by diabetes and developmental delay but without epilepsy (Hattersley and Ashcroft 2005).

ND may be due to an inherited mutation but is more frequently sporadic in origin, resulting from *de novo* mutations (Gloyn, Cummings et al. 2004; Edghill, Gloyn et al. 2007). Mutations in the insulin gene (*INS*), the glucokinase gene (*GCK*) and the forkhead box P3 gene (*FOXP3*) have been reported to cause ND. However, the most common form of neonatal diabetes is caused by heterozygous activating mutations in the genes *KCNJ11* and *ABCC8*, which encode the two subunits (Kir6.2 and SUR1, respectively) of the ATP-sensitive potassium (K_{ATP}) channel (Gloyn, Cummings et al. 2004; Babenko, Polak et al. 2006).

About 30% of cases of TNDM and 50% of PNDM cases result from gain-of-function mutations in Kir6.2, the pore-forming subunit of the K_{ATP} channel. These mutations impair the ability of intracellular ATP to close the channel (Proks, Antcliff et al. 2004; Ashcroft 2007). Consequently, the K_{ATP} current is increased leading to hyperpolarisation of the β -cell and reduced insulin secretion. This in turn leads to low plasma insulin levels and a diabetic phenotype. Because the K_{ATP} channel is also expressed in muscle and brain, mutation of Kir6.2 (and more rarely of SUR1) can also result in DEND or iDEND (Hattersley and Ashcroft 2005).

Patients with PNDM, who have little or no endogenous insulin secretion, were originally treated with lifelong insulin injections. Identification of a K_{ATP} channel mutation in these patients has enabled about 90 % of them to switch to sulphonylurea tablet medication, an appreciably important lifestyle change (Pearson, Flechtner et al. 2006). Sulphonylureas bind to the SUR1 subunit of the pancreatic β -cell K_{ATP} channel and close the channel in an ATP-independent manner, thereby stimulating insulin secretion. Most patients carrying a K_{ATP} channel mutation are treated with the sulphonylurea glibenclamide (0.4–0.8 mg/kg/day), often at doses that are considerably higher than those used for the treatment of type 2 diabetes (Pearson, Flechtner et al. 2006). Sulphonylureas are known to close K_{ATP} channels in nerve, muscle and brain tissue as well as β -cells (Akrouh, Halcomb et al. 2009). This may explain the improvement of neurological

defects and motor dysfunction that have been observed in patients with iDEND syndrome after initiating sulphonylurea therapy (Slingerland, Nuboer et al. 2006; Koster, Cadario et al. 2008).

4.1.2 MOUSE MODELS EXPRESSING AN ACTIVATING MUTATION OF THE K_{ATP} CHANNEL IN β -CELLS

The function of genes has traditionally been studied by observing the effects of spontaneous or rarely occurring mutations in the whole organism. Recent advances in molecular biology have made it possible to manipulate the mouse genome by introducing, modifying or knocking out a specific gene *in vivo*. This enables the role of gene products in physiological processes to be studied. Mice with extra or altered copies of a gene in their genome are termed transgenic mice. The Cre/loxP recombination system has emerged as one of the most widely used transgenic technologies, as it allows for both tissue and temporal expression control of the transgene (Lewandoski 2001).

In order to better understand the genetic etiology of ND, improve treatment and better predict disease progression, I studied two transgenic mouse models of ND expressing the most common mutation causing iDEND syndrome (Kir6.2-V59M) (Ashcroft 2007). They were generated by the Cre/loxP recombination method and consisted of

- 1) the β -V59M mouse that expresses the Kir6.2-V59M transgene exclusively and constitutively in the pancreatic β -cell (Girard, Wunderlich et al. 2009)
- 2) the ib-V59M mouse which expresses an inducible form of the same transgene specifically in pancreatic β -cells.

In order to create β -V59M mice, the ROSA mouse line was crossed with the RIP-Cre mouse line (Fig. 4.1).

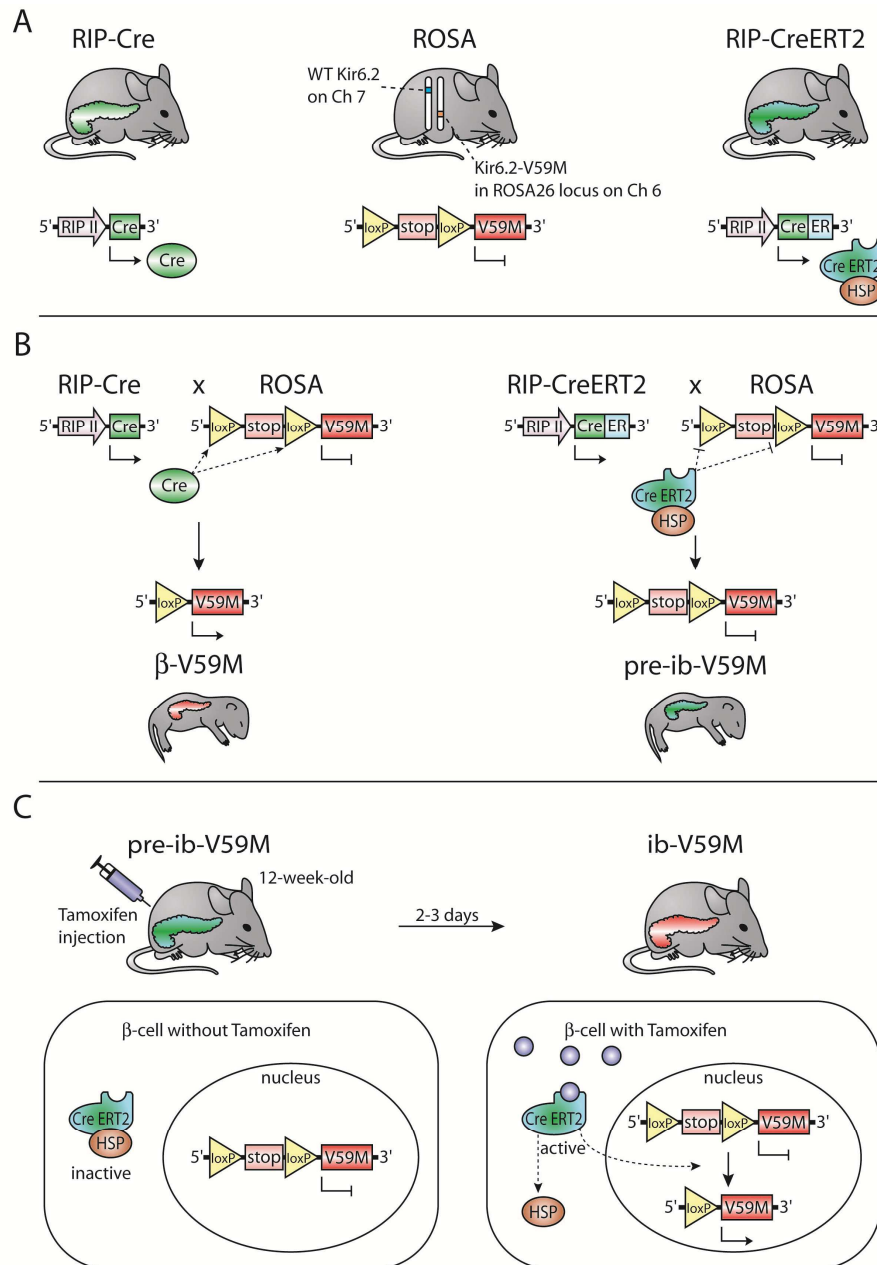


Fig. 4.1 Generation of β -V59M and ib-V59M mice

A RIP-Cre mice (left) express Cre recombinase (Cre), driven by the rat insulin promoter II (RIP II), exclusively in the β -cell. In ROSA mice (middle) the Kir6.2-V59M transgene (V59M) lies downstream of a loxP-flanked stop sequence, preventing expression of the mutant channel protein. The transgene was integrated into the ROSA26 locus on chromosome 6 (Ch 6). The mouse also carries two copies of the wild-type gene (on chromosome 7) and is hemizygous for the V59M transgene. RIP-CreERT2 mice (right) express RIP-driven CreERT2 which is produced by fusing Cre to a modified estrogen ligand-binding domain (ER) that renders the recombinase inactive. **B** β -V59M mice (left), which express the V59M transgene constitutively in β -cells, were generated by crossing RIP-Cre mice with ROSA mice. In β -cells of β -V59M mice Cre excises the loxP-flanked stop sequence, permitting expression of the transgene. Crossing RIP-CreERT2 mice with ROSA mice give rise to pre-ib-V59M mice (right) in which the V59M transgene has not yet been induced and is thus switched off. **C** In the absence of ligand CreERT2 is inactive and confined to the cytoplasm by binding to heat-shock proteins (HSP). Upon injection of synthetic steroids (tamoxifen) - but not in the presence of endogenous steroids - HSP are displaced from CreERT2, so allowing it to translocate to the nucleus and activate the transgene.

ROSA mice carry the Kir6.2-V59M transgene downstream of a transcriptional stop sequence, which is flanked by two loxP sequences in the same orientation (Girard, Wunderlich et al. 2009). The stop cassette prevents the transgene from being transcribed. Thus ROSA mice are expected to have a wild-type phenotype. Introducing the transgene into the ROSA26 locus endows it with the ubiquitously active ROSA26 promoter. As the Kir6.2-V59M gene was inserted by the means of homologous recombination these mice have a single copy of the mutant gene, and are thus hemizygous, carrying two copies of the wild-type gene and one copy of the mutant gene. RIP-Cre mice express Cre recombinase under the control of the rat insulin promoter II and were obtained from Pedro Herrera (Herrera 2000). The presence of Cre recombinase alone should not affect the expression of endogenous genes and these mice are also expected to show a wild-type phenotype. In the F1 progeny of ROSA and RIP-Cre mice, the Kir6.2-V59M transgene and Cre recombinase are brought together. Cre recombinase then recognises the two loxP sites and recombination results in excision of the intervening stop sequence and consequently activation of the transgene.

To generate ib-V59M mice, the ROSA mouse line was first mated with the RIP-CreERT2 mouse line (kindly provided by Dr Yuval Dor). RIP-CreERT2 mice express a Cre recombinase, driven by the RIPII promoter, which is fused to a modified ligand binding domain of the estrogen receptor ERT2. In the absence of ligand, the CreERT2 is bound to heat-shock proteins (HSP) and confined to the cytoplasm, hence Cre is inactive. These mice, in which the transgene has not been induced, are called pre-ib-V59M mice. Expression of transgene requires the presence of the synthetic estrogen receptor ligands tamoxifen or 4-hydroxytamoxifen (endogenous estrogen is ineffective). Binding of tamoxifen to CreERT2 then displaces HSPs and Cre is translocated to the nucleus (Feil, Brocard et al. 1996; Indra, Warot et al. 1999).

The transgenic mouse model, generated by the Cre/loxP recombination system has several advantages over classical gene knock-in or knockout techniques:

- 1) depending on the tissue specificity of the Cre recombinase promoter the target gene (Kir6.2-V59M) can be activated in a particular cell type. Use of the insulin promoter, for example, targets expression to insulin-producing cells like the pancreatic β -cell. This makes it possible to study the single role of β -cells in the development of ND and to eliminate effects on glucose homeostasis mediated by the expression of Kir6.2-V59M in other cell types such as neurons or muscle cells.
- 2) The tamoxifen-sensitive Cre recombinase in ib-V59M mice enables the target gene to be activated at a particular stage of development. This can overcome the risk of a systemic compensation for the genetic abnormality or the loss of precursor cell populations which can both lead to failure of the mouse to develop properly (Albanese, Hult et al. 2002).

Both β -V59M and ib-V59M mice, however, are not free of caveats: the V59M transgene is not regulated by its endogenous promoter, which may lead to a different expression level of the transgene. The V59M mouse models should simulate the heterozygous state of the K_{ATP} channel mutations causing ND in patients. It was therefore important that the transgene was not overexpressed. The mouse Rosa26 locus has proven useful for genetic modification as it can be targeted with high efficiency and endows the transgene with its weak ROSA promoter. Indeed, it could be shown that expression levels of mutant and wild-type Kir6.2 subunit were similar in β -V59M mice (Girard, Wunderlich et al. 2009). It is also unlikely that transgenic expression of a mutant Kir6.2 subunit will result in an increase in K_{ATP} channel density, because assembly of the channel is regulated by the level of the SUR1 subunit and the Kir6.2 subunit will not traffic to the cell membrane without SUR1 (Aguilar-Bryan and Bryan 1999).

In *Xenopus* oocytes the Kir6.2-V59M mutation increases the intrinsic open probability of the K_{ATP} channel and thereby indirectly renders it less sensitive to inhibition by ATP (Proks, Antcliff

et al. 2004). It was therefore hypothesised that K_{ATP} channels in β -cells of V59M mice would show reduced sensitivity to ATP. Consequently, increased glucose metabolism would fail to close the channel and no plasma membrane depolarisation would occur, resulting in impaired Ca^{2+} influx.

In the first part of this chapter I tested this hypothesis by measuring glucose-stimulated $[Ca^{2+}]_i$ in whole islets isolated from β -V59M mice. In addition, islets from RIP-Cre mice, ROSA mice and wild-type mice (which express neither the V59M transgene nor Cre recombinase) were used. All these mice were predicted to have a wild-type response. This work has been published (Girard, Wunderlich et al. 2009).

In the second part of this chapter I investigated glucose-stimulated $[Ca^{2+}]_i$ responses both in whole islets and in individual β -cells within intact islets isolated from *ib*-V59M islets. In contrast to the β -V59M mouse, which display severe diabetes by 5-weeks of age, the *ib*-V59M mouse should develop in the absence of hyperglycemia and therefore be free of possible complications such as impaired β -cell development, tissue damage or general changes in cellular metabolism (Rolo and Palmeira 2006; Giaccari, Sorice et al. 2009). In these islets the effect of glibenclamide treatment *in vivo* and *in vitro* was also examined.

4.2 METHODS

4.2.1 MOLECULAR BIOLOGY

4.2.1.1 GENOTYPING OF B-V59M MICE AND IB-V59M MICE

Genotypes were identified by PCR using genomic DNA isolated from ear biopsies (DNeasy Blood and Tissue Kit; QIAGEN). The presence of the Kir6.2-V59M mutant gene was confirmed using the following set of primers: P1, 5'-AAAGTCGCTCTGAGTTGTTATC-3'; P2, 5'-GATATGAAGTACTGGGCTCTT-3'; and P3, 5'-GCATCGCCTTCTATCGCCT-3'. These amplify a 590-bp amplicon from the WT ROSA26 allele but a 460-bp product from the Kir6.2-V59M targeted allele. The presence of the RIP-Cre gene was confirmed by the amplification of an approximately 230-bp product, using forward ACGAGTGATGAGGTTCGCA and reverse ATGTTTAGCTGGCCCAAATGT primers. For both genes, PCR conditions were 94°C for 3 min, followed by 45 cycles of 94°C for 30 sec, 57°C for 45 sec, 72°C for 90 sec. This was followed by a final extension of 72°C for 10 min.

4.2.1.2 GENERATION OF TRANSGENIC MICE

4.2.1.3 B-V59M MICE

β -V59M mice were generated by crossing the ROSA mouse line with the RIP-Cre mouse line. Wild-type, ROSA and RIP-Cre littermates were used as controls.

4.2.1.4 IB-V59M MICE

ib-V59M mice were generated by first mating the ROSA mouse line with the RIP-CreERT2 mouse line, giving rise to pre-ib-V59M mice. In order to activate Kir6.2-V59M in pre-ib-V59M mice, mice

were given a single subcutaneous injection of tamoxifen at 12 weeks of age (0.4 ml of 20 mg/ml tamoxifen in corn oil). This was carried out by Dr Kenju Shimomura. Approximately 2-3 days after injection, the blood glucose level rose significantly, indicating activation of the transgene. Mice in which the Kir6.2-V59M transgene has been induced are called ib-V59M mice.

In some ib-V59M mice, a placebo pellet was implanted once the blood glucose had reached a concentration of > 20 mM. In other mice, a 21-day slow-release pellet containing 0.25 mg glibenclamide was implanted to facilitate glycemic control. For a mouse of 25 g and a release duration of 14-21 days this corresponds to a dose of 0.5-0.7 mg/kg/day. This is within the range used to treat patients with ND (Pearson, Flechtner et al. 2006).

Islets were either isolated from 14-week-old ib-V59M mice following a 10-day period of glibenclamide therapy or from 14-week-old control mice. Control mice consisted of pre-ib-V59M which were sham-injected (injected with only vehicle) and wild-type mice: both did not receive glibenclamide therapy (Fig. 4.2). Isolated Islets were incubated overnight in culture medium without glibenclamide. On the day of the experiment both control and ib-V59M islets were divided into two groups. One group of islets was treated with 2 μ M glibenclamide throughout the whole experiment, including a 30 min pre-incubation in 2 mM glucose. The other group was not exposed to glibenclamide. A glibenclamide concentration of 2 μ M is sufficient to fully close the wild-type K_{ATP} channel (Gromada, Dissing et al. 1995; Proks, Reimann et al. 2002).

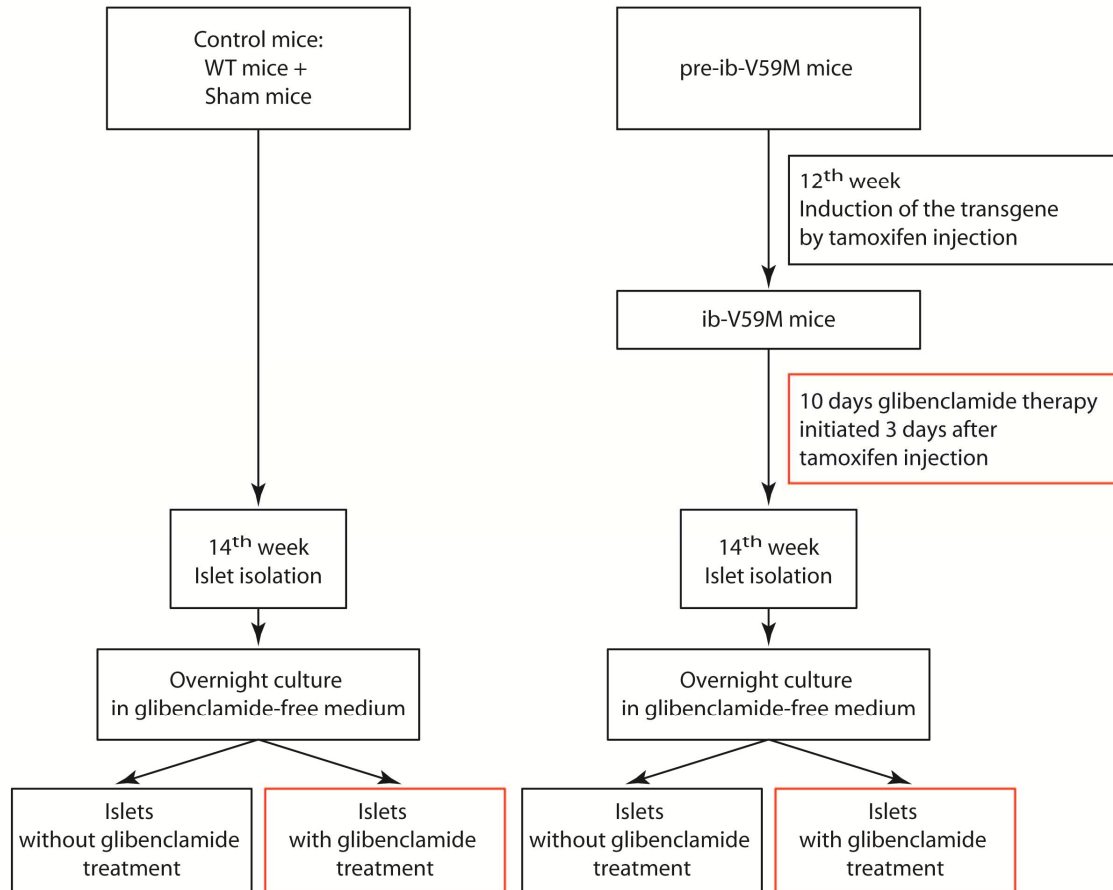


Fig. 4.2 Flow chart of procedures executed on control or pre-ib-V59M mice and islets

Control mice include wild-type mice and sham-injected mice. The latter were injected with vehicle (corn oil) and showed no activation of the V59M transgene.

4.2.2 MEASUREMENTS OF $[Ca^{2+}]_i$ IN β -V59M OR IB-V59M ISLETS

A number of parameters of the glucose- or tolbutamide-stimulated $[Ca^{2+}]_i$ responses were measured as shown in figure 4.3. Basal Ca^{2+} was measured by averaging $[Ca^{2+}]_i$ during the total time of exposure to 2 mM glucose (~10 min). Application of 20 mM glucose often led to an initial fall in $[Ca^{2+}]_i$. This nadir was determined as the difference between basal Ca^{2+} and the minimum $[Ca^{2+}]_i$ before the Ca^{2+} peak produced by 20 mM glucose solution. Experiments where no nadir was observed were excluded from the analysis. Since β -V59M and ib-V59M islets did not display a glucose-stimulated peak in $[Ca^{2+}]_i$, their $[Ca^{2+}]_i$ response was measured at 4.3 min (for β -V59M and control islets) or 6.6 min (for ib-V59M or control islets) after glucose addition. These times

corresponded to the average time it took each set of control islets to show a glucose-evoked Ca^{2+} peak. The secondary increase in $[\text{Ca}^{2+}]_i$ produced by 20 mM glucose was measured 10.5 min (for β -V59M and control islets) or 15 min (for *ib*-V59M and control islets) after glucose addition.

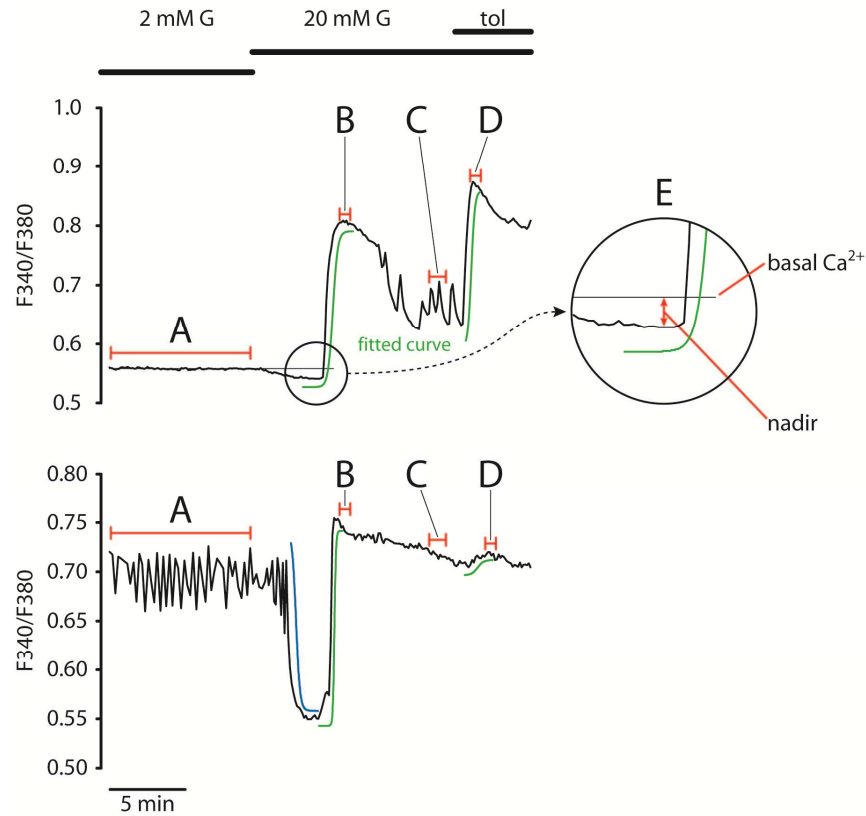


Fig. 4.3 Example of representative $[\text{Ca}^{2+}]_i$ responses recorded from a pancreatic islet in the absence (top trace) of and in the presence (bottom trace) of glibenclamide

Islets were stimulated with 20 mM glucose and tolbutamide (tol) and the parameters analysed are shown. **A** Time over which basal $[\text{Ca}^{2+}]_i$ in 2 mM glucose was measured. **B** Time over which the peak $[\text{Ca}^{2+}]_i$ response in 20 mM glucose was measured. Measurements were taken at 4.3 min (β -V59M islets) and 6.6 min (*ib*-V59M islets) after the beginning of glucose application. Approximately 60 data points, corresponding to 30 sec, were averaged. **C** Time over which the secondary increase in $[\text{Ca}^{2+}]_i$ was measured. Measurements were taken at 10.5 min (β -V59M islets) and 15 min (*ib*-V59M islets) after the beginning of glucose application. About 120 data point, corresponding to 1 min, were averaged. **D** Time over which the peak $[\text{Ca}^{2+}]_i$ response in 0.2 mM tolbutamide (tol) was measured. Tolbutamide (0.2 mM or 0.5 mM) was either applied in the presence of 2 mM or 20 mM glucose as indicated in the experiment. **E** The nadir of initial fall in $[\text{Ca}^{2+}]_i$ (nadir) was calculated as the difference between basal Ca^{2+} , measured in A, and the minimum $[\text{Ca}^{2+}]_i$ before the glucose-stimulated Ca^{2+} peak. The Ca^{2+} increase upon glucose and tolbutamide stimulation was fitted to the Hill equation 1 (green lines). The transient fall in $[\text{Ca}^{2+}]_i$ (bottom trace) was fitted to the Hill equation 2 (blue line). The fitted curves have been offset relative to the original data for clarity. Parameters for both equations are given in Chapter 2.

The $[Ca^{2+}]_i$ responses in 20 mM glucose and the peak response produced by application of tolbutamide (either 0.2 mM or 0.5 mM as indicated in experiment) were measured by averaging approximately 60 data points over about 30 sec. The increase in $[Ca^{2+}]_i$ elicited by stimulation with either 20 mM glucose or tolbutamide (if present) was fitted to the Hill equation 1 (parameters are given in Chapter 2) and the maximum rate of change in $[Ca^{2+}]_i$ was reported.

When islets were incubated with glibenclamide their basal $[Ca^{2+}]_i$ was elevated. In these islets, application of 20 mM glucose produced a transient fall in $[Ca^{2+}]_i$ which returned to levels similar to basal $[Ca^{2+}]_i$ (Fig. 4.3 bottom Ca^{2+} trace). This decrease in $[Ca^{2+}]_i$ was fitted to the Hill equation 2 (parameters are given in Chapter 2) and the maximal decrease rate of change in $[Ca^{2+}]_i$ was reported.

4.3 RESULTS

4.3.1 $[Ca^{2+}]_i$ RESPONSES OF β -V59M ISLETS COMPARED WITH CONTROL ISLETS

Changes in $[Ca^{2+}]_i$ in β -V59M islets and control (wild-type, ROSA and RIP-Cre) islets were measured in response to glucose. To demonstrate that the islets were healthy, the K_{ATP} channel was functional and there was no impairment of Ca^{2+} signalling downstream of the K_{ATP} channel, all islets were subsequently exposed to a maximally effective concentration (0.2 mM) of the K_{ATP} channel blocker tolbutamide. Only islets that showed a clear response to either glucose or tolbutamide were included in the analysis.

No significant differences in basal $[Ca^{2+}]_i$ were observed between β -V59M and control islets (Fig. 4.4 A-E). In most islets, 20 mM glucose produced an initial small decrease in $[Ca^{2+}]_i$ reflecting glucose-stimulated Ca^{2+} uptake into intracellular stores, (Chow, Lund et al. 1995). There was no significant difference in the size of the nadir between control and β -V59M islets (Fig. 4.4 F). In control islets, the initial decrease was followed by a large increase in $[Ca^{2+}]_i$ (Fig. 4.4 A, C, D, E). In contrast, 20 mM glucose produced a significantly slower and smaller $[Ca^{2+}]_i$ increase in β -V59M islets (Fig. 4.4 B, E). The difference in the glucose-stimulated increase in $[Ca^{2+}]_i$ between control and β -V59M islets was even more evident when changes in $[Ca^{2+}]_i$ were expressed as the maximal rate of the rise of the response (Fig. 4.4 G). However, there was no significant difference in $[Ca^{2+}]_i$ between the genotypes when $[Ca^{2+}]_i$ was measured at 10.5 min after glucose application. The mean change in $[Ca^{2+}]_i$ and the maximum rate of rise of the response evoked by 0.2 mM tolbutamide plus 2 mM glucose did not show any significant difference. (Fig. 4.4 F, G).

Some islets displayed an oscillatory $[Ca^{2+}]_i$ response to low glucose. It is possible that these oscillations were produced by α -cells which are active in low glucose.

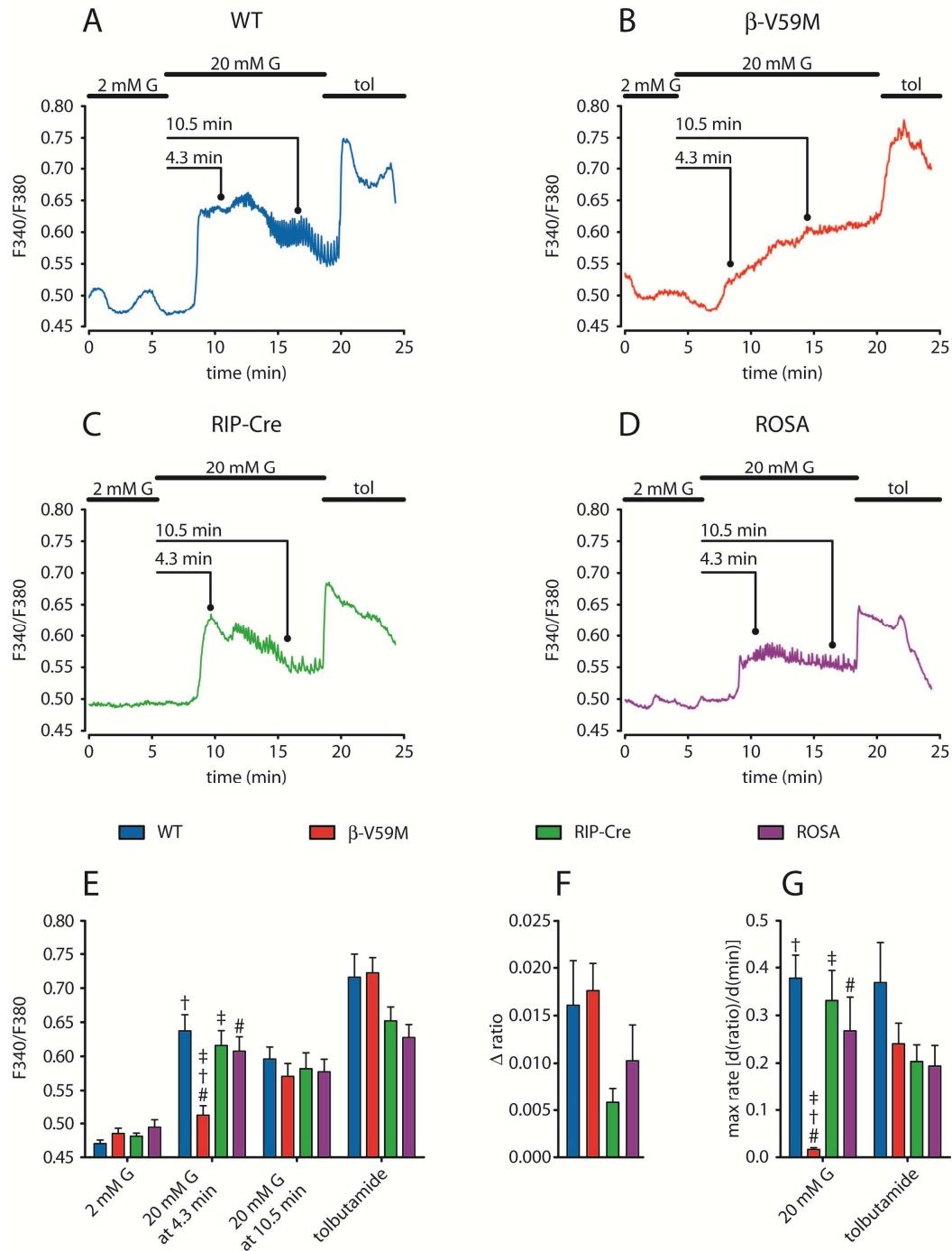


Fig. 4.4 Changes in $[Ca^{2+}]_i$ of 5-week-old β -V59M and control islets upon stimulation with 20 mM glucose or 0.2 mM tolbutamide plus 2 mM glucose

A-D Representative Ca^{2+} traces recorded from wild-type (WT), β -V59M, RIP-Cre and ROSA islets. **E** Mean data ($\pm$ SEM), showing the basal $[Ca^{2+}]_i$ response to 2 mM glucose, 20 mM glucose (measurement taken 4.3 min and 10.5 min after the beginning of stimulation) and 0.2 mM tolbutamide plus 2 mM glucose. **F** Mean data ($\pm$ SEM), showing the nadir of the initial fall in $[Ca^{2+}]_i$ for WT ($n = 8$), β -V59M ($n = 9$), RIP-Cre ($n = 9$) and ROSA ($n = 5$) islets. **G** Mean data ($\pm$ SEM) of the maximum rate of change in $[Ca^{2+}]_i$ on the initial application of 20 mM glucose and 0.2 mM tolbutamide plus 2 mM glucose. Data, if not stated otherwise, were obtained from 3 WT mice (10 islets), 2 β -V59M mice (9 islets), 3 RIP-Cre mice (12 islets) and 3 ROSA mice (11 islets). Significance was tested using a One-Way ANOVA test and the Bonferroni post-test. Means with the same symbols are significantly different. $\#$, $\dagger$, $\ddagger$ $p < 0.05$.

4.3.2 INDUCTION OF THE V59M TRANSGENE IN PRE-IB-V59M MICE

Following tamoxifen injection the blood glucose level of pre-ib-V59M mice became significantly elevated after a few days, indicating that the V59M transgene had been expressed (Fig. 4.5). These mice are now referred to as ib-V59M mice. The induction of the transgene was associated with a marked loss of β -cells after 1-2 weeks of high blood glucose (Dr Kenju Shimomura personal communication). However, islets were preserved when the mice were treated with glibenclamide. Therefore, I used islets isolated from glibenclamide-treated mice to test the effects of the V59M transgene on the $[Ca^{2+}]_i$ response to glucose.

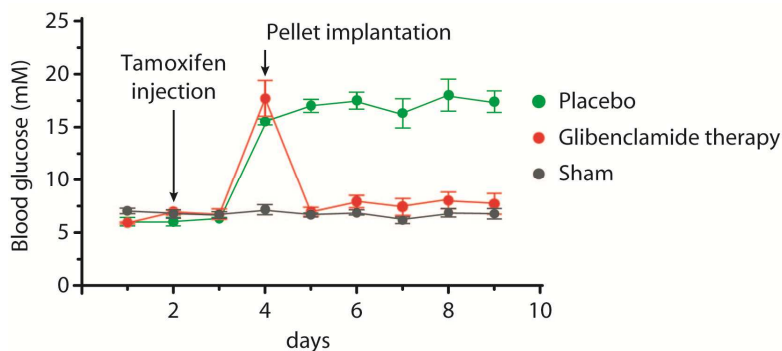


Fig. 4.5 Blood glucose levels of transgenic mice following induction of the V59M transgene with and without glibenclamide therapy

A single injection of tamoxifen in pre-ib-V59M mice (arrowed) elevated glucose levels to about 20 mM within the next 2 days. Upon subcutaneous implantation of a glibenclamide pellet (arrowed) glucose levels decreased immediately whereas implantation of a placebo pellet had no effect. The term Sham was used for pre-ib-V59M mice which have been injected with vehicle (corn oil): no elevation of blood glucose was observed in these mice. Data is expressed as mean $\pm$ SEM of 5 placebo mice, 5 glibenclamide therapy mice and 6 Sham mice. Data by courtesy of Dr Kenju Shimomura.

4.3.3 $[Ca^{2+}]_i$ RESPONSES OF WILD-TYPE AND SHAM ISLETS

I first examined the $[Ca^{2+}]_i$ responses of islets isolated from wild-type and pre-ib-V59M mice injected with vehicle alone (sham-injected mice). Islets from 14-week-old wild-type and sham-injected mice displayed a similar magnitude and time course of their $[Ca^{2+}]_i$ in response to glucose or 0.5 mM tolbutamide plus 20 mM glucose (Fig. 4.6).

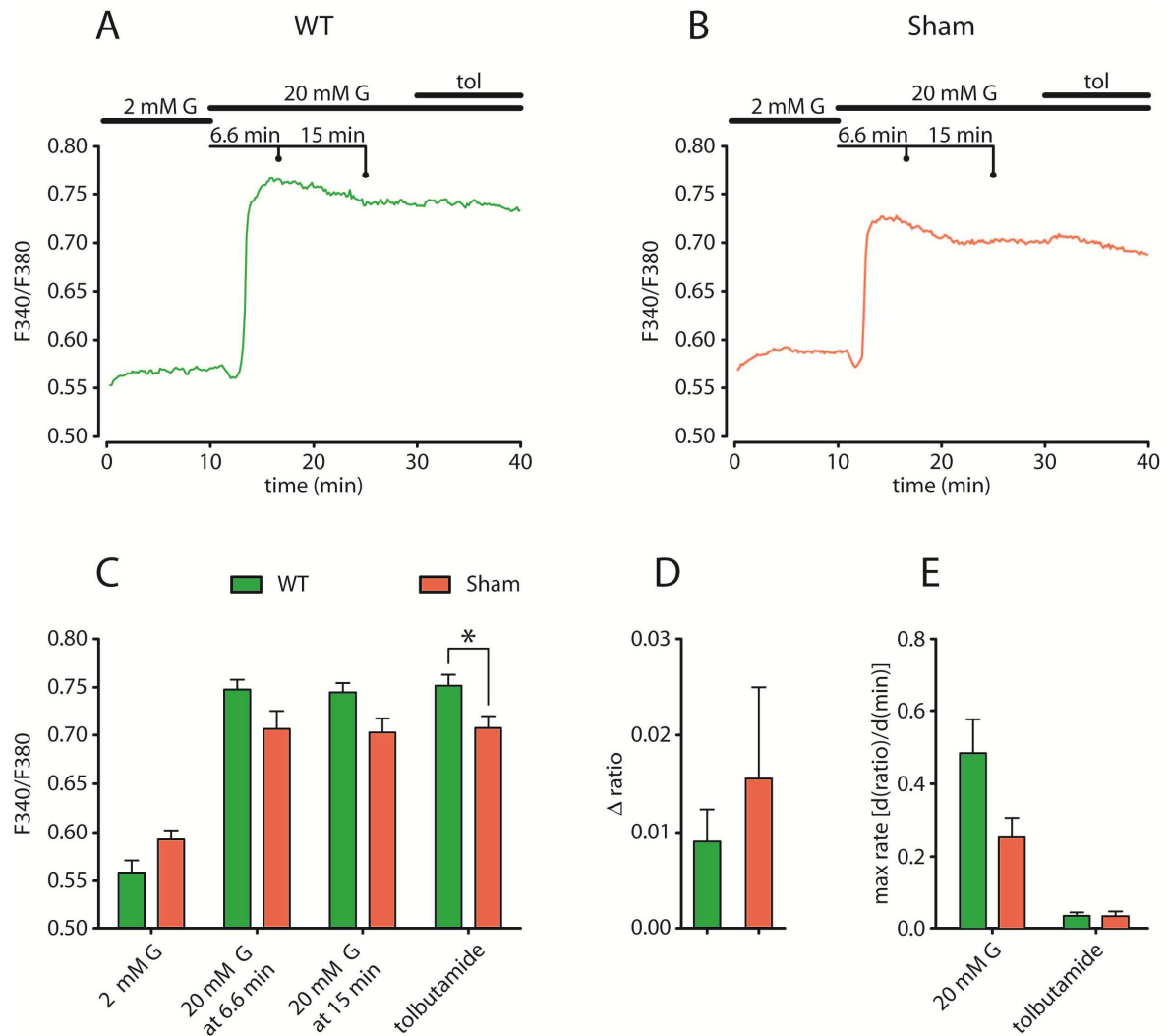


Fig. 4.6 Changes in $[Ca^{2+}]_i$ recorded from wild-type and Sham islets upon stimulation with 20 mM glucose and 0.5 mM tolbutamide plus 20 mM glucose

A-B Representative $[Ca^{2+}]_i$ responses recorded from wild-type (A) and Sham (B) islets. **C** Mean data ($\pm$ SEM), showing the basal $[Ca^{2+}]_i$ response to 2 mM glucose, 20 mM glucose (measurement taken 6.6 min and 15 min after the beginning of stimulation), and 0.5 mM tolbutamide plus 20 mM glucose. **D** Mean data ($\pm$ SEM), showing the nadir of initial fall in $[Ca^{2+}]_i$. **E** Mean data ($\pm$ SEM) of the maximum rate of change in $[Ca^{2+}]_i$ on the initial application of 20 mM glucose and 0.5 mM tolbutamide plus 20 mM glucose. Data were obtained from two WT mouse (8 islets) and one Sham mouse (4 islets). Significance was tested using an unpaired t-test. $p < 0.05$.

There was no significant difference in basal $[Ca^{2+}]_i$ when wild-type and sham islets were exposed to 2 mM glucose (Fig. 4.6 A-C). The response of both types of islets to 20 mM glucose was characterised by an initial small decrease in $[Ca^{2+}]_i$ (nadir) followed by a robust increase in $[Ca^{2+}]_i$. There was no significant difference in the $[Ca^{2+}]_i$ response measured at 6.6 or 15 min after

application of 20 mM glucose (Fig. 4.6 B), the size of the nadir (Fig. 4.6 D) or the maximum rate of change in $[Ca^{2+}]_i$ upon stimulation with 20 mM glucose or with 0.5 mM tolbutamide plus 20 mM glucose (Fig. 4.6 E) between wild-type and sham islets. However, the peak $[Ca^{2+}]_i$ response to 0.5 mM tolbutamide plus 20 mM glucose was significantly elevated in wild-type islets compared with sham islets.

Since wild-type and sham islets showed the same glucose-stimulated $[Ca^{2+}]_i$ responses their data were pooled together, and termed control islets in the rest of this chapter.

4.3.4 $[Ca^{2+}]_i$ RESPONSES OF IB-V59M ISLETS

Control and ib-V59M islets both exhibited a similar basal Ca^{2+} level in 2 mM glucose (Fig. 4.7 A, C). Since ib-V59M mice were treated with glibenclamide this finding suggests that overnight incubation without glibenclamide is sufficient to render islets free of the effects of the drug.

The magnitude of the fall in $[Ca^{2+}]_i$ produced by 20 mM glucose (nadir) was similar in both sets of mice (Fig. 4.7 F). Glucose (20 mM) elicited a robust increase in $[Ca^{2+}]_i$ in control islets but failed to produce an initial rapid increase in ib-V59M islets: only a slow and gradual increase could be observed (Fig. 4.7 A, C). Mean data for $[Ca^{2+}]_i$ at 6.6 min after the start of glucose stimulation, and for the maximum increase of $[Ca^{2+}]_i$ rate are given in figure. 4.7 E, G. Even after 15 min of glucose stimulation, $[Ca^{2+}]_i$ in ib-V59M islets was still significantly lower than in control islets. Subsequent addition of 0.5 mM tolbutamide plus 20 mM glucose only caused a small increase of $[Ca^{2+}]_i$ in both groups (Fig. 4.7 E, G).

The maximum rate of change in $[Ca^{2+}]_i$ provides a mean to quantitatively compare the slow and small increase of $[Ca^{2+}]_i$ upon glucose stimulation between β -V59M and ib-V59M islets. As expected, there was a significant difference ($P = 0.02$) in the maximum $[Ca^{2+}]_i$ rate between β -V59M (0.017 ± 0.004 ratio/min) and ib-V59M islets (0.006 ± 0.001 ratio/min).

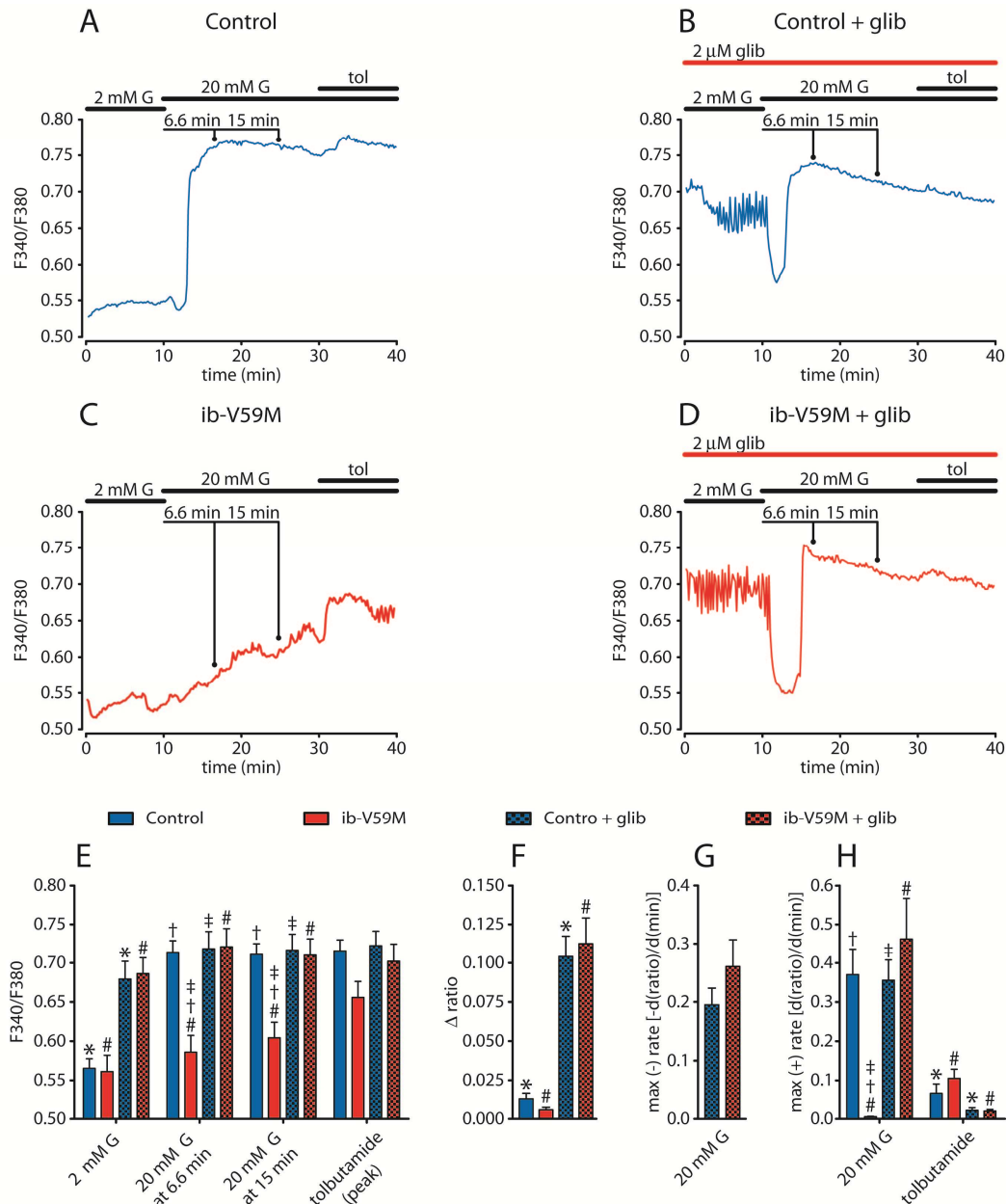


Fig. 4.7 Effect of 2 μM glibenclamide on $[\text{Ca}^{2+}]_i$ in control and ib-V59M islets upon stimulation with 20 mM glucose and 0.5 mM tolbutamide plus 20 mM glucose

Control islets were isolated from wt or sham injected mice. **A-D** Representative Ca^{2+} traces from control and ib-V59M islets incubated without glibenclamide (A, C) and with glibenclamide (B, D). **E** Mean data ($\pm$ SEM), showing the response to 2 mM glucose, 20 mM glucose (measurement taken 6.6 min and 15 min after the beginning of stimulation) and 0.5 mM tolbutamide plus 20 mM glucose. **F** Mean data ($\pm$ SEM), showing the initial nadir in $[\text{Ca}^{2+}]_i$ for control ($n = 8$), ib-V59M ($n = 5$), control + glib ($n = 10$) and ib-V59M + glib ($n = 14$). **G** Mean data ($\pm$ SEM) for the maximum decrease rate of change in $[\text{Ca}^{2+}]_i$ in response to 20 mM glucose. **H** Mean data ($\pm$ SEM) for the maximum increase rate in $[\text{Ca}^{2+}]_i$ in response to 20 mM glucose or 0.5 mM tolbutamide plus 20 mM glucose. Number of islets analysed for the response to tolbutamide: control ($n = 8$), ib-V59M ($n = 11$), control + glib ($n = 8$), ib-V59M + glib ($n = 5$). Data, if not stated otherwise, were obtained from 3 control mice (12 islets - glib, 11 islet + glib) and 3 ib-V59M mice (11 islets - glib, 14 islets + glib). Significance was tested using a two-way ANOVA test and the Bonferroni post-test. Control vs. ib-V59M ($\dagger$), control vs. control + glib ($*$), ib-V59M vs. control + glib ($\ddagger$) and ib-V59M vs. ib-V59M + glib ($\#$) are significantly different at $\dagger$, $*$, $\ddagger$, $\#$ $p < 0.05$.

4.3.5 EFFECT OF GLIBENCLAMIDE ON CALCIUM RESPONSES IN IB-V59M ISLETS

In order to mimic the situation of patients with neonatal diabetes on glibenclamide therapy, islets were pre-incubated with 2 μ M glibenclamide and the perfusion solution was also supplemented with 2 μ M glibenclamide. Exposure to 2 μ M glibenclamide resulted in similar $[\text{Ca}^{2+}]_i$ responses in both control and ib-V59M islets (Fig. 4.7 B, D).

Control islets exposed to 2 mM glucose plus 2 μ M glibenclamide showed a significant elevation of $[\text{Ca}^{2+}]_i$ (Fig. 4.7 B). In most islets, marked oscillations in Ca^{2+} levels were observed. Stimulation with 20 mM glucose initially caused a large fall in $[\text{Ca}^{2+}]_i$ which subsequently recovered back to a level similar to that found in 2 mM glucose. The Ca^{2+} levels remained elevated throughout glucose stimulation as demonstrated by measurements taken at 6.6 min and 15 min (Fig. 4.7 E). There was no further increase in $[\text{Ca}^{2+}]_i$ in response to 0.5 mM tolbutamide plus 20 mM glucose. This result is consistent with all sulphonylurea-binding sites being already occupied by glibenclamide.

As in control islets, exposure of ib-V59M islets to 2 mM glucose plus 2 μ M glibenclamide led to a marked elevation of $[\text{Ca}^{2+}]_i$ (Fig. 4.7 D) and continuous Ca^{2+} oscillations. Application of 20 mM glucose produced a substantial transient fall in $[\text{Ca}^{2+}]_i$ which returned to a level similar to that in 2 mM glucose. $[\text{Ca}^{2+}]_i$ showed a sustained elevation in 20 mM glucose and there was no difference in the magnitude when measured at 6.6 min and 15 min after the beginning of stimulation. Like control islets, 0.5 mM tolbutamide plus 20 mM glucose failed to increase $[\text{Ca}^{2+}]_i$ in ib-V59M islets. Mean data are shown in figure 4.7 E.

Resting $[\text{Ca}^{2+}]_i$, measured at 2 mM glucose in the presence of 2 μ M glibenclamide was not different between control and ib-V59M islets but was markedly greater than that in the absence of glibenclamide (Fig. 4.7 E). There was no significant difference in the size of the nadir (Fig. 4.7 F), the maximum decrease rate (Fig. 4.7 G) or the maximum increase rate of $[\text{Ca}^{2+}]_i$ (Fig. 4.7 H)

recorded in control and ib-V59M islets, exposed to glibenclamide, upon stimulation with glucose. Likewise, the $[Ca^{2+}]_i$ responses measured at 6.6 min and 15 min after glucose addition showed no significant difference (Fig. 4.7 E). Similarly, there was no difference in the mean $[Ca^{2+}]_i$ (Fig. 4.7 E) or the maximum rate of rise of the response evoked by tolbutamide between the two groups (Fig. 4.7 H).

Glibenclamide treatment significantly increased the $[Ca^{2+}]_i$ response to 20 mM glucose stimulation in ib-V59M islets (comparing Fig. 4.7 C with Fig. 4.7 D). However, control islets showed no further increase in $[Ca^{2+}]_i$ upon glucose stimulation (comparing Fig. 4.7 A with Fig. 4.7 B), suggesting that $[Ca^{2+}]_i$ was already maximal (Fig. 4.7 E).

4.3.6 $[Ca^{2+}]_i$ RESPONSES OF INDIVIDUAL β -CELLS IN IB-V59M ISLET

The data presented thus far demonstrate the $[Ca^{2+}]_i$ responses of the whole islets. In order to study the Ca^{2+} responses of individual β -cells within the islet and see whether they exhibit synchronous oscillations, calcium imaging using confocal microscopy was performed. Figure 4.8 shows the representative responses of three β -cells, distributed across the islet, and the average response of the whole islet.

In control islets, β -cells displayed a stable basal Ca^{2+} trace at 2 mM glucose (Fig. 4.8 A). In 20 mM glucose all three control β -cells responded simultaneously with a robust Ca^{2+} influx, followed by short in-phase oscillations of Ca^{2+} (small window). The Ca^{2+} peak was preceded by an initial lowering of the Ca^{2+} signal. Similar to measurements in whole islets, 0.5 mM tolbutamide plus 20 mM glucose failed to raise Ca^{2+} levels further in most β -cells.

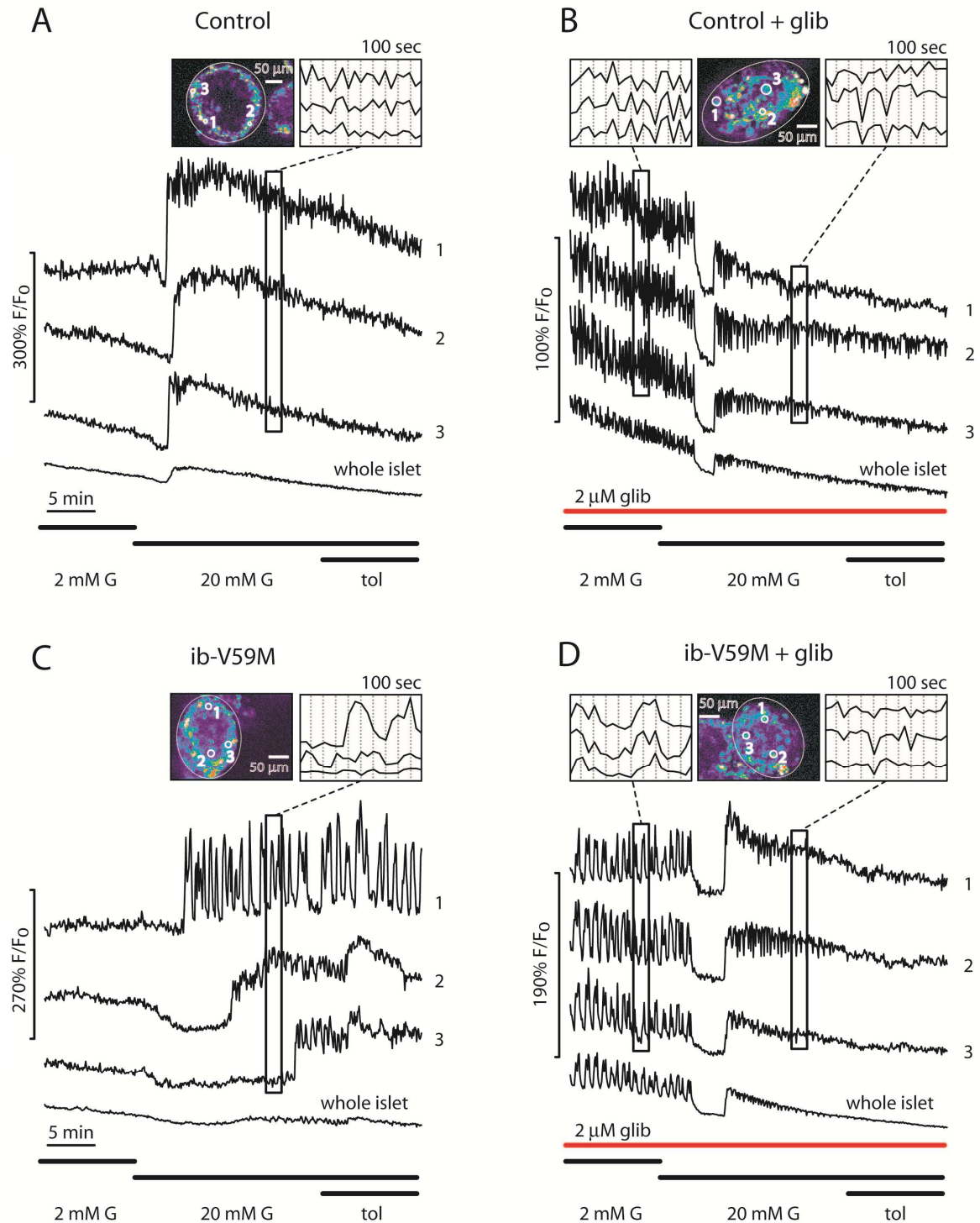


Fig. 4.8 Effect of glibenclamide on Ca^{2+} oscillations in individual β -cells within control and ib-V59M islets upon stimulation with 20 mM glucose and 0.5 mM tolbutamide plus 20 mM glucose.

Control islets were isolated from wt or sham injected mice. Representative β -cells are marked on images of the islets (top) and $[\text{Ca}^{2+}]_i$ responses of these cells are shown below. Insets (top) show amplified sections of Ca^{2+} oscillations. **A-D** Representative Ca^{2+} signals recorded simultaneously from three individual β -cells (as indicated) and the average response of the whole islet (lowest trace) for control (A), and ib-V59M (C) islets. The effect of 2 μM glibenclamide on Ca^{2+} traces are shown for control β -cells (B) and ib-V59M β -cells (D). Fluorescence was measured over a period of 40 min. Data were obtained from 3 control mice (7 islets - glib, 8 islet + glib) and 3 ib-V59M mice (8 islets - glib, 16 islets + glib).

β -cells from ib-V59M islets also exhibited stable Ca^{2+} levels at 2 mM glucose (Fig. 4.8 C). Exposure to 20 mM glucose produced an initial fall in the Ca^{2+} level, that was prolonged in some β -cells, and, interestingly, was followed by a steep increase in $[\text{Ca}^{2+}]_i$. However, these individual responses were not coordinated and in most β -cells they were of a smaller magnitude than in control β -cells. Consequently, they were not resolved at the whole islet level. Furthermore, the Ca^{2+} oscillations evoked by 20 mM glucose were not synchronous across the islet (small window). Application of 0.5 mM tolbutamide plus 20 mM glucose had only a small effect on the Ca^{2+} signal.

4.3.7 $[\text{Ca}^{2+}]_i$ RESPONSES OF INDIVIDUAL B-CELLS IN IB-V59M ISLET EXPOSED TO GLIBENCLAMIDE

In the presence of 2 μM glibenclamide, β -cells of control islets exhibited vigorous and synchronous oscillations at 2 mM glucose (Fig. 4.8 B, left window). Switching to 20 mM glucose produced a simultaneous fall in $[\text{Ca}^{2+}]_i$ in all β -cells that quickly returned to the same level as that observed in 2 mM glucose. After this, oscillations resumed in-phase between all three β -cells but their amplitude gradually declined (right window). Ca^{2+} oscillations, both at 2 mM and 20 mM glucose were also resolved at the whole islet level. Tolbutamide had no effect on the Ca^{2+} trace. This was expected because glibenclamide would have already closed K_{ATP} channels.

β -cells in ib-V59M islets produced a glucose-stimulated $[\text{Ca}^{2+}]_i$ response similar to β -cells in control islets when exposed to glibenclamide (Fig. 4.8 D). In 2 mM glucose marked Ca^{2+} oscillations were observed that were synchronised between β -cells (left window). A transient fall in $[\text{Ca}^{2+}]_i$ was triggered by 20 mM glucose, but $[\text{Ca}^{2+}]_i$ quickly recovered to levels similar to that in 2 mM glucose. β -cells stimulated with 20 mM glucose displayed synchronous oscillations (right window) that gradually petered out. These Ca^{2+} oscillations in 2 mM and 20 mM glucose were also observed at the whole islet level. Similar to control β -cells, tolbutamide had no effect in ib-V59M β -cells.

This experiment demonstrates that application of glibenclamide to ib-V59M islets produces marked, in-phase oscillations of their β -cells in 2 mM glucose. Interestingly, it also restores the synchronous Ca^{2+} oscillations of individual β -cells upon glucose stimulation.

4.3.8 NUMBER OF B-CELLS RESPONDING TO GLUCOSE

In order to assess the effect of glibenclamide therapy in ib-V59M mice on the viability of their β -cells, the number of β -cells within the confocal plane responding to glucose stimulation with an increase their $[\text{Ca}^{2+}]_i$ (experiment of Fig. 4.8) was counted.

Figure 4.9 shows that exposure of both control and ib-V59M islets to 2 μM glibenclamide had no effect on the number of β -cells responding to 20 mM glucose. Interestingly, unlike β -V59M islets (Girard, Wunderlich et al. 2009), ib-V59M islets (with or without glibenclamide treatment) did not show a reduction in the number of β -cells responding to high glucose compared to control islets (Fig. 4.9). This indicates that glibenclamide therapy is successful in preserving the viability of β -cells.

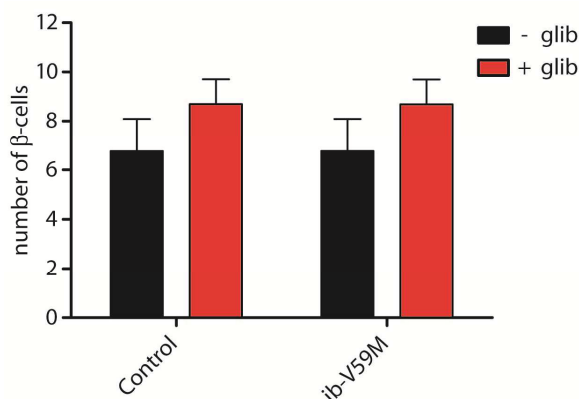


Fig. 4.9 Number of β -cells in control and ib-V59M islets responding to 20 mM glucose

The glucose stimulation lasted for 20 min. β -cells were counted within the confocal plane ($\sim 20 \mu\text{m}$) in the presence and absence of glibenclamide. Data represents mean $\pm$ SEM and were obtained from 3 control mice (7 islets - glib, 8 islet + glib) and 3 ib-V59M mice (8 islets - glib, 16 islets + glib) from experiment Fig. 4.8. Significance was tested using a two-way ANOVA test and no significance was found.

4.4 DISCUSSION

4.4.1 CONSTITUTIVE EXPRESSION OF KIR6.2-V59M

Wild-type islets responded to glucose stimulation with a robust influx of Ca^{2+} . Ca^{2+} influx is mainly controlled by voltage-gated Ca^{2+} channels (Mears 2004), which open upon membrane depolarisation triggered by K_{ATP} channel closure.

In contrast to wild-type islets, 20 mM glucose produced a significantly slower and smaller $[\text{Ca}^{2+}]_i$ increase in β -V59M islets. This is because the K_{ATP} channels in β -V59M β -cells exhibited a significant reduction in the ATP sensitivity compared to wild-type β -cells: half maximal inhibition (IC_{50}) was produced by approximately 270 μM ATP for β -V59M β -cells versus approximately 25 μM ATP for wild-type β -cells (Girard, Wunderlich et al. 2009). Even at supra-physiological concentrations of ATP (10 mM), there was a substantial fraction of unblocked current in β -V59M β -cells (~7% for β -V59M vs. ~0.5% for wild-type) (Girard, Wunderlich et al. 2009). This suggests that V59M K_{ATP} channels are never completely closed in their native cellular environment. These data suggest that the absence of a robust Ca^{2+} increase in V59M mutant islets upon stimulation with high glucose can be attributed to the failure of the channel to be shut by elevated ATP and so elicit a depolarisation mediated Ca^{2+} influx. Tolbutamide (0.2 mM) blocked about 82% of the β -V59M currents (Girard, Wunderlich et al. 2009). Since there was a comparable $[\text{Ca}^{2+}]_i$ response to tolbutamide in control and β -V59M islets, this blockade seems to be sufficient to evoke depolarisation of the plasma membrane and trigger Ca^{2+} influx.

In order to demonstrate that the reduced glucose-stimulated $[\text{Ca}^{2+}]_i$ response observed in β -V59M islets was specific for the expression of the V59M transgene, islets isolated from ROSA and RIP-Cre mice were used as controls. Unspecific effects could arise from insertion of a transgene (Lewandoski 2001) or Cre-mediated recombination at pseudo-loxP sites that may result in undesired functional consequences (Lee, Ristow et al. 2006; Fex, Wierup et al. 2007). However,

$[Ca^{2+}]_i$ responses stimulated by both glucose and tolbutamide were similar in ROSA and RIP-Cre islets compared with wild-type islets.

Figure 4.10 shows that basal, first- and second-phase insulin secretion upon stimulation with glucose was significantly reduced in β -V59M islets compared to wild-type islets (Girard, Wunderlich et al. 2009). Loss of first-phase insulin secretion has been reported to be an early characteristic of type II diabetes (Rorsman, Eliasson et al. 2000). The noticeable lack of first-phase insulin secretion was mirrored by the nearly complete absence of the initial Ca^{2+} influx in β -V59M upon glucose stimulation.

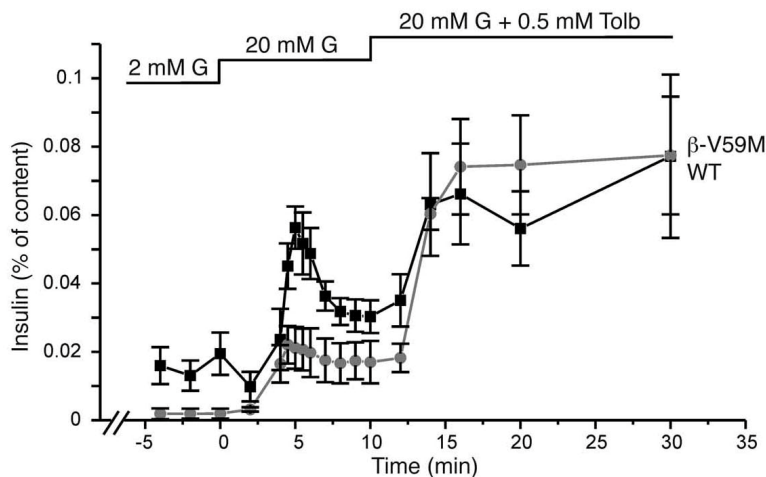


Fig. 4.10 Insulin secretion from perfused islets isolated from 5-week-old wild-type (WT, squares) or β -V59M (circles) islets in response to 20 mM glucose and 0.5 mM tolbutamide plus 20 mM glucose

Basal insulin secretion and both first and second-phase insulin secretion stimulated by glucose were impaired in β -V59M islets. In contrast, tolbutamide evoked the same amount of secretion. Secretion was normalized to insulin content and is expressed as mean $\pm$ SEM. Figure taken from Girard et al. (Girard, Wunderlich et al. 2009).

In wild-type islets the first-phase insulin secretion was followed by a sustained second-phase during which the secretion rate dropped to a level that was intermediate between the peak first-phase and basal secretion rates. In β -V59M islets this decline in insulin secretion rate during the second-phase was less prominent and the magnitude of secretion between first-phase and second-phase was not significantly different. Interestingly, the secondary increase in $[Ca^{2+}]_i$,

corresponding to the second-phase of insulin secretion, was similar in β -V59M and control islets. It is conceivable that this secondary increase in $[Ca^{2+}]_i$ was responsible for sustaining second-phase insulin secretion in β -V59M islets.

4.4.2 INDUCIBLE EXPRESSION OF KIR6.2-V59M

ib-V59M islets isolated from mice treated with glibenclamide and then cultured overnight in the absence of sulphonylurea had a resting $[Ca^{2+}]_i$ in 2 mM glucose similar to islets isolated from control mice. Thus overnight culture is sufficient to remove all glibenclamide from the islet.

In ib-V59M islets stimulation with 20 mM glucose only produced a slow and gradual increase in $[Ca^{2+}]_i$. This is because the K_{ATP} channels in ib-V59M mice displayed reduced ATP sensitivity ($IC_{50} = 275$) (Dr Kenju Shimomura, personal communication) which was similar to that found in β -V59M mice ($IC_{50} = 270$).

In ib-V59M islets, tolbutamide produced no further increase in $[Ca^{2+}]_i$ when added on top of 20 mM glucose. This was probably because the plasma membrane was already fully depolarised.

4.4.3 EFFECTS OF GLIBENCLAMIDE ON IB-V59M ISLETS

Closure of K_{ATP} channels by glibenclamide elicited a comparable $[Ca^{2+}]_i$ response in both control and ib-V59M islets upon stimulation with glucose or tolbutamide. This suggests that V59M gene activation does not have an effect on Ca^{2+} signalling downstream of the K_{ATP} channel. The mean Ca^{2+} level in 2 mM glucose was similar to the peak $[Ca^{2+}]_i$ response recorded at 20 mM glucose.

Interestingly, insulin secretion data on perfused ib-V59M islets treated with glibenclamide, kindly provided by Dr Kenju Shimomura, revealed a nearly 4-fold elevation of basal insulin secretion upon 20 mM glucose stimulation (Fig. 4.11). This increase in insulin secretion, despite no further increase of $[Ca^{2+}]_i$, can be attributed to the amplifying pathway as described by Henquin et al. (Henquin 2000). Under these conditions, glucose still increases insulin secretion in a concentration-dependent manner. My results suggest that this amplifying pathway was also operational in ib-V59M islets.

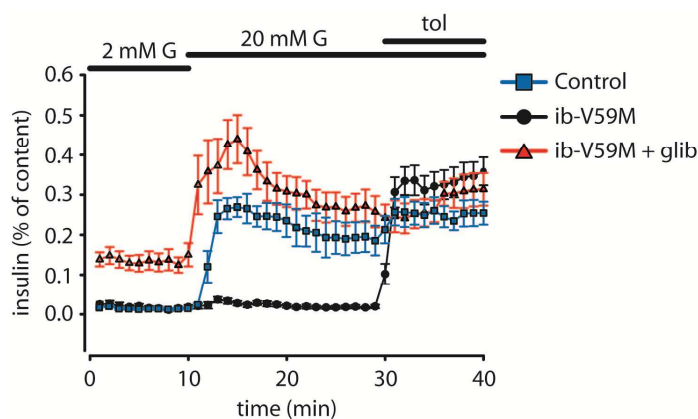


Fig. 4.11 Insulin secretion from perfused control and ib-V59M islets, isolated from 14-week-old mice

Islets were stimulated with 2 mM glucose, then 20 mM glucose, and finally 0.5 mM tolbutamide plus 20 mM glucose. Experiment were carried out in the absence (control, ib-V59M) or presence (ib-V59M + glib) of 2 μ M glibenclamide. Secretion was normalized to insulin content. Data were obtained from 4 control mice and 8 ib-V59M mice and are expressed as mean $\pm$ SEM. Data by courtesy of Dr Kenju Shimomura.

In both wild-type and ib-V59M islets, 20 mM glucose, in the absence of glibenclamide, produced an initial fall in $[Ca^{2+}]_i$. This is believed to be produced by an ATP-dependent removal of Ca^{2+} (by SERCA pumps) from the cytoplasm into intracellular stores (Chow, Lund et al. 1995; Tengholm, Hellman et al. 2001). When islets of both genotypes were incubated with glibenclamide, their basal $[Ca^{2+}]_i$ was elevated and 20 mM glucose produced a much greater decrease in $[Ca^{2+}]_i$ than was observed in the absence of glibenclamide. A similar glucose-stimulated $[Ca^{2+}]_i$ response was observed in islets or β -cells from SUR1 knockout mice which lack K_{ATP} channels, or in wild-type islets or β -cells in which the K_{ATP} channel was blocked by tolbutamide (Nenquin, Szollosi et al.

2004; Dufer, Haspel et al. 2009). Dufer et al. showed that in SUR1 knockout β -cells or β -cells in which the K_{ATP} channel was blocked the glucose-stimulated transient fall in $[Ca^{2+}]_i$ was independent of SERCA pumps and could still be observed when these pumps were pharmacologically blocked (Dufer, Haspel et al. 2009). In this paper it was proposed that the transient fall of $[Ca^{2+}]_i$ is caused by glucose activating the electrogenic Na^+/K^+ -ATPase, which leads to a transient hyper-polarisation of the plasma membrane. During the hyper-polarisation voltage-dependent Ca^{2+} channels close, whereas the clearance of cytosolic Ca^{2+} is facilitated by the action of the plasma membrane Na^+/Ca^{2+} exchanger, leading to a decrease in $[Ca^{2+}]_i$.

There was no difference in the magnitude or in its rate of fall in $[Ca^{2+}]_i$ produced by 20 mM glucose in the presence or absence of glibenclamide. Assuming that the activity of the Ca^{2+} pumps was the same in β -V59M and control islets, it could be suggested that glucose metabolism and the generation of ATP was unimpaired in transgenic V59M islets.

4.4.4 IMAGING OF SINGLE B-CELLS IN IB-V59M ISLETS

Calcium imaging enables the oscillatory behaviour of $[Ca^{2+}]_i$ in single β -cells to be studied and also allows investigation of whether all β -cells fail to respond to glucose stimulation in ib-V59M islets. This could help to provide an estimate of the extent to which the gene is activated - is it mosaic or is it uniformly expressed in all β -cells?

In control islets, Ca^{2+} oscillations were synchronised, as previously reported (Gilon, Shepherd et al. 1993; Zhang, Galvanovskis et al. 2008; Girard, Wunderlich et al. 2009). In contrast, β -cells of ib-V59M islets responded to 20 mM glucose with non-synchronised Ca^{2+} oscillations across the islet. This is consistent with observations made for β -V59M islets, which have been published in Girard et al. (Girard, Wunderlich et al. 2009). It was noticeable that most ib-V59M β -cells responded much later than control β -cells and in an uncoordinated fashion to the glucose

stimulus. Additionally, the magnitude of the $[Ca^{2+}]_i$ response in most ib-V59M β -cells was smaller than in control β -cells. Altogether this led to a lack of the initial increase of $[Ca^{2+}]_i$ in the averaged signal for the whole islet. This is consistent with ratiometric measurements of $[Ca^{2+}]_i$ in ib-V59M islets (Fig. 4.7).

The observed decline of the signal over the course of the whole experiment is likely to be due to bleaching of the fluo-4 dye. In order to minimise the effect of bleaching the laser intensity and the exposure time were kept to a minimum.

4.4.5 COMPARISON OF THE GLUCOSE-STIMULATED $[Ca^{2+}]_i$ RESPONSES BETWEEN B-V59M AND IB-V59M ISLETS

In contrast to β -V59M islets, glucose-stimulated insulin secretion in ib-V59M islets was characterised by a complete loss of both first-phase and second-phase (Fig. 4.11). This was paralleled by a $[Ca^{2+}]_i$ response which exhibited a nearly complete loss of the initial increase and a significantly reduced secondary increase in $[Ca^{2+}]_i$ upon glucose stimulation. Additionally, the maximum rate of increase of $[Ca^{2+}]_i$ observed after stimulation with glucose was significantly lower in ib-V69M than β -V59M islets.

4.4.6 POSSIBLE MOLECULAR MECHANISM FOR THE SLOW INCREASE IN $[Ca^{2+}]_i$ OBSERVED IN BOTH B-V59M AND IB-V69M ISLETS UPON GLUCOSE STIMULATION

Although the electrophysiological data suggested that the V59M K_{ATP} channel was never completely shut under physiological conditions (Girard, Wunderlich et al. 2009), individual β -cells responded to glucose stimulation and a secondary increase in $[Ca^{2+}]_i$ was observed in both β -

V59M and ib-V59M islets. This raises further questions about the nature of the rise in $[Ca^{2+}]_i$ in V59M transgenic islets upon glucose stimulation.

An intuitive explanation would be that the transgene was not expressed at a sufficient level in all β -cells to prevent glucose-dependent depolarization. Unfortunately, it was not possible to monitor expression of the transgene in individual cells, because the GFP reporter gene was expressed at very low levels (Girard, Wunderlich et al. 2009). However, it seems reasonable to assume that the transgene might be activated in a mosaic fashion as reported by others (Lewandoski 2001). Findings by Rocheleau et al., however, would argue against an increase in $[Ca^{2+}]_i$ mediated by β -cells with low or no expression of the transgene (Rocheleau, Remedi et al. 2006). Their study demonstrated that mice expressing a loss-of-function K_{ATP} channel mutation in 70% of their β -cells exhibited nearly normal Ca^{2+} signalling, being uniformly hyperpolarised under resting conditions and depolarised on glucose addition. The authors suggested that the 30% of β -cells that had normal K_{ATP} channels were sufficient to provide enough current (via gap junctional coupling) to hyperpolarise those cells that lacked functional K_{ATP} channels. This idea is supported by the fact that hyperpolarisation of one β -cell could attenuate or even stop the electrical activity of nearby coupled β -cells (Speier, Gjinovci et al. 2007). Based on these reports, it may be reasonable to suggest that the V59M transgene only has to be expressed in a fraction of β -cells in order to account for the failure of the whole β -V59M and ib-V59M islet to respond to glucose stimulation with a rise in $[Ca^{2+}]_i$: the gain-of-function mutation should keep the β -cells hyperpolarised, and clamp the electrically coupled adjacent β -cells at the resting potential.

The findings presented above suggest that a K_{ATP} channel-independent process might underlie the secondary increase in $[Ca^{2+}]_i$ found in both β -V59M and ib-V59M islets. A possible mechanism might involve, for example, the release of Ca^{2+} from intracellular stores mediated by nicotinic acid adenine dinucleotide phosphate (NAADP). Studies by Masgrau et al. showed that NAADP is produced upon glucose stimulation in clonal pancreatic β -cells (MIN6) and elicits an

increase in $[Ca^{2+}]_i$ at low NAADP concentrations (Masgrau, Churchill et al. 2003). NAADP is believed to release Ca^{2+} from lysosome-related stores (Calcraft, Ruas et al. 2009). In normal β -cells NAADP-induced Ca^{2+} release is proposed to activate Ca^{2+} -activated nonselective cation channels (TRPM4/5) at the plasma membrane. This would contribute to membrane depolarisation and so enhance Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Cheng, Beck et al. 2007). In V59M islets expressing constitutively active K_{ATP} channels the plasma membrane would not depolarise and Ca^{2+} entry through voltage-dependent Ca^{2+} channels would be prevented. However, glucose might still produce NAADP-mediated Ca^{2+} mobilisation: the Ca^{2+} signal observed in either β -V59M or ib-V59M islets might reflect this release. Studies using agents such as GPN (glycyl-L-phenylalanine- β -naphthylamide) or bafilomycin that selectively block NAADP-induced Ca^{2+} release (Galione 2006) might help determine if this idea is valid.

Girard et al. showed that the number of β -cells in β -V59M islets displaying glucose-stimulated Ca^{2+} increase was greatly reduced compared to wild-type islets (Girard, Wunderlich et al. 2009). Furthermore, the reduction in β -cell number was confirmed by histological studies. It was also noticeable that fewer islets could be isolated from β -V59M mice and most of those islets did not survive overnight culture. These data contrast with the findings on ib-V59M islets isolated from mice treated with glibenclamide, where the number of glucose-responsive β -cells was not found to be different to wild-type β -cells. It was found that these ib-V59M islet also show no difference in insulin content or number of β -cells in comparison to wild-type islets (Dr Kenju Shimomura personal communication). This suggests that the reduction of β -cells in β -V59M mice is probably not mediated by K_{ATP} channel activation, which, for example, has been found to play a role in promoting death of dopaminergic midbrain neurons (Liss, Haeckel et al. 2005). Rather it is likely that the reduction of β -cells in β -V59M islets is due to the persistent hyperglycaemia of these mice (Rolo and Palmeira 2006; Giaccari, Sorice et al. 2009).

4.5 SUMMARY

A gain-of-function mutation (V59M) in the gene encoding the Kir6.2 subunit of the K_{ATP} channel causes ND in humans. A transgenic mouse model expressing V59M in β -cell recapitulates the disease in humans, and exhibits a reduction of both first- and second-phase insulin secretion *in vitro*. Measurement of $[Ca^{2+}]_i$ on islets, isolated from mice constitutively expressing the transgene (β -V59M), demonstrated that this secretion profile was paralleled by a complete loss of the initial rise in $[Ca^{2+}]_i$ upon glucose stimulation. However, islets from ROSA mice (carrying a non expressing V59M Kir6.2 gene alone) or from RIP-Cre mice (expressing Cre recombinase alone) showed no impairment in their $[Ca^{2+}]_i$ responses. If the V59M expression is induced in adult animals (ib-V59M mice) and glycemic control was ensured by *in vivo* glibenclamide administration, glucose-stimulated islets displayed a loss of the initial rise in $[Ca^{2+}]_i$ and a reduction in secondary phase. Calcium imaging revealed a loss of synchronicity of Ca^{2+} oscillations between β -cells within the mutant islets, but showed no difference in the number of β -cells responding to glucose compared with control islets. *In vitro* treatment on V59M islets elevated basal $[Ca^{2+}]_i$ and restored both first- and second-phase rise of $[Ca^{2+}]_i$, as well as the synchronous $[Ca^{2+}]_i$ response between individual β -cells.

5 A MOUSE MODEL OF HYPERINSULINISM OF INFANCY EXPRESSING A LOSS-OF-FUNCTION MUTATION (E1506K) IN THE SUR1 SUBUNIT OF THE K_{ATP} CHANNEL

5.1 INTRODUCTION

5.1.1 HYPERINSULINEMIA OF INFANCY

Hyperinsulinemia of Infancy (HI), also named persistent hyperinsulinemic hypoglycemia of infancy or congenital hyperinsulinism, is a heterogeneous genetic disorder affecting 1 in 40,000– 50,000 people (James, Kapoor et al. 2009). It is characterised by dysregulated insulin secretion, that results in hypoglycemia (< 3 mM blood glucose) in both the fed and fasted state (Hussain, Blankenstein et al. 2007). Mechanistically, hypoglycemia is caused by unregulated insulin secretion from pancreatic β -cells. This deprives the brain of energy, and when untreated can lead to hypoglycemic brain injury and mental retardation (Hussain, Blankenstein et al. 2007). The clinical manifestations of HI are very diverse, ranging from completely asymptomatic, through a mild, drug-responsive condition, to a severe drug-resistant disease which may require total pancreatectomy. Patients undergoing a total pancreatectomy have a high risk of developing post-pancreatectomy diabetes mellitus and pancreatic exocrine insufficiency.

In most cases HI occurs sporadically, although a much rarer congenital form of HI has also been documented (Flanagan, Clauin et al. 2009). To date, 50% of the known cases of HI can be accounted for by mutations in one of seven genes (James, Kapoor et al. 2009). A rare recessive form of HI is due to mutations in the 3-hydroxyacyl-coenzyme-A dehydrogenase gene (*HADH*) (Clayton, Eaton et al. 2001), and dominant forms of HI are caused by activating mutations in the glutamate dehydrogenase gene (*GLUD1*) (Stanley, Lieu et al. 1998), the glucokinase gene (*GCK*) (Glaser, Kesavan et al. 1998), the hepatocyte nuclear factor 4a gene (*HNF4A*) (Pearson, Boj et al. 2007) and the monocarboxylate transporter 1 gene (*SLC16 A1*) (Otonkoski, Jiao et al. 2007).

The most common cause of HI, however, is inactivating mutations in the *ABCC8* and *KCNJ11* genes which encode the SUR1 and Kir6.2 subunits of the pancreatic β -cell K_{ATP} channel, respectively (Thomas, Cote et al. 1995; Thomas, Ye et al. 1996). Mutations in the K_{ATP} channel causing HI can be inherited either recessively or dominantly.

To date, more than 150 mutations in *ABCC8* (SUR1) including missense, frameshift, nonsense, insertion or deletion mutations have been found to be associated with HI. Fewer mutations have been reported in *KCNJ11* (Kir6.2) (Flanagan, Clauin et al. 2009). SUR1 and Kir6.2 mutations can be classified by the type of defect they cause in K_{ATP} channel function (Ashcroft 2005). Class I mutations reduce the K_{ATP} channel density by altering channel biogenesis or increasing the turnover rate of the mutated protein (Crane and Aguilar-Bryan 2004). Additionally, these mutations may affect the trafficking of the channel leading to absent or reduced surface expression (Nestorowicz, Inagaki et al. 1997; Cartier, Conti et al. 2001). Class II mutations are characterised by the failure of MgADP to activate the channel when metabolism is low; as a result, the K_{ATP} channel remains shut, depolarising the plasma membrane and consequently triggering the influx of Ca^{2+} through voltage-gated Ca^{2+} channels, causing $[Ca^{2+}]_i$ to rise, and lead to continuous insulin secretion (Ashcroft 2005).

Huopio et al. first reported a dominant form of HI that is caused by a missense SUR1 mutation (E1506K). They found that expression of the mutation in the heterozygous form was sufficient to trigger the disease (Huopio, Reimann et al. 2000; Huopio, Otonkoski et al. 2003). Patients carrying this mutation were prone to develop glucose intolerance and insulin deficiency in early adulthood and diabetes later in life. Clinically, the phenotype was variable with manifestation of HI either within the first few hours after birth, between 5 to 7 months later, or even after the fourth year of life (Huopio, Reimann et al. 2000; Pinney, MacMullen et al. 2008; Vieira, Bergamin et al. 2009). Most patients showed a good response to diazoxide treatment.

The E1506K mutation is located in nucleotide-binding domain 2 of SUR1 (Fig. 1.5), and electrophysiological analysis of heterogeneously expressed K_{ATP} channels in *Xenopus* oocytes revealed that the current through the channel was reduced due to impaired MgADP activation. There was no obvious change in ATP inhibition at Kir6.2 (Huopio, Reimann et al. 2000). In accordance with the clinical findings, the K_{ATP} channel opener diazoxide produced a small but noticeable increase in channel activity. Like other dominant K_{ATP} mutations causing HI, SUR1-E1506K does not interfere with channel assembly or trafficking and allows for normal K_{ATP} channel formation at the plasma membrane (Pinney, MacMullen et al. 2008).

To further investigate the molecular mechanisms of HI and the transition from neonatal HI to diabetes in later life, Professor Markku Laakso designed a knock-in mouse model expressing the SUR1-E1506K mutation. The mouse was generated at the *Institut Clinique de la Souris* (ICS) in Strasbourg and consists of a knock-in mouse on a C57BL/6 background. The endogenous *ABCC8* gene was replaced with the mutant *ABCC8* gene by homologous recombination. Hence, every cell in the animal carries the SUR1-E1506K mutation. Furthermore, since the endogenous promoter and cis-regulatory elements were not altered, normal expression patterns and normal regulation of gene expression are expected. In this chapter mice which are heterozygous for the SUR1-E1506K mutation are termed het-E1506K. Homozygous mice are termed hom-E1506K.

Since the SUR1-E1506K mutation impairs MgADP activation of the K_{ATP} channel, it is hypothesised that the channel remains shut even at low plasma glucose concentrations, thereby producing maintained depolarisation of the plasma membrane, Ca^{2+} influx and insulin secretion. In the following study, this hypothesis was tested by characterising the insulin secretion profile, Ca^{2+} signalling and electrophysiological properties of SUR1-E1506K mutant islets.

5.2 METHODS

5.2.1 BUFFERS/MEDIA

5.2.1.1 ELECTROPHYSIOLOGY

- EC Buffer-II (standard whole cell): 120 mM NaCl, 3.6 mM KCl, 2 mM NaHCO₃, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 2.5 mM CaCl₂, 5 mM HEPES, 5 mM glucose, 20 mM tetraethylammonium chloride (TEA-Cl), pH 7.4 with NaOH.
- Pipette Solution: 125 mM potassium glutamate, 10 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 0.05 mM EGTA, 3 mM Mg-ATP, 5 mM HEPES and 0.1 mM cAMP, (pH 7.1 with KOH)

5.2.2 MOLECULAR BIOLOGY

5.2.2.1 GENOTYPING SUR1 E1506K MICE

Genotypes were identified by PCR using genomic DNA isolated from ear biopsies (DNeasy Blood and Tissue Kit; QIAGEN). SUR1 E1506K mice were genotyped using the primer pair P1, 5'-GAGGGTGAACCAGCAATGTCAGAG-3' and P2, 5'-CTCCATACCACAGTGTTCCTTGAGC-3'. PCR products of 184 bp for wild-type and 293 for the knock-in allele were amplified under the following conditions: 3 min at 94°C followed by initial 2 cycles of 94°C for 1 min, 62°C 1 min, and 72°C for 1 min, followed by 30 cycles of 94°C for 30 sec, 62°C 30 sec, and 72°C for 30 sec. The PCR was finalised by an extension 72°C for 7 min.

5.2.3 ELECTROPHYSIOLOGY

The development of the patch-clamp technique by Neher and Sakmann in the late 1970s has provided one of the most powerful methods for studying of ion channel function. Membrane currents or membrane potentials were recorded from superficial cells within an intact pancreatic islet, using the standard whole-cell technique. Patch pipettes were pulled from borosilicate glass and filled with Pipette Solution. The extracellular solution was EC buffer-II, which contained tetraethylammonium chloride to block outward delayed rectifying potassium currents, and was continuously perfused. Experiments were carried out at 32-34°C.

Currents were recorded every 0.02 ms (I-V protocol) or 0.01 ms ($h\infty$ protocol) using a software-controlled patch-clamp amplifier (EPC9, HEKA electronics). The software Pulse and Pulsefit (HEKA) were used to control the membrane potential and to digitise and record the currents. A 4/p leak subtraction was applied (Bezánilla and Armstrong 1977). Further analysis was carried out by the computer.

5.2.4 MEASUREMENTS OF $[Ca^{2+}]_i$ IN E1506K ISLETS

A number of parameters of the glucose- or tolbutamide-stimulated $[Ca^{2+}]_i$ responses were measured as shown in figure 5.1. Since E1506K islets displayed marked Ca^{2+} oscillations in 2 mM glucose solution, basal Ca^{2+} was measured by averaging $[Ca^{2+}]_i$ during the total time of exposure to 2 mM glucose (~14 min). Additionally, $[Ca^{2+}]_i$ was also measured for the final 3.5 min of exposure to 2 mM glucose before changing to 10 mM glucose. The peak increase in $[Ca^{2+}]_i$ produced by application of 10 mM glucose or 0.2 mM tolbutamide plus 10 mM glucose, was measured by averaging $[Ca^{2+}]_i$ over 30 sec, or 3.5 min, as shown. The increase in $[Ca^{2+}]_i$ elicited by stimulation with either 20 mM glucose or tolbutamide (if present) was fitted to the Hill equation 1 (parameters are given in Chapter 2) and the maximum rate of change in $[Ca^{2+}]_i$ was reported. In

order to quantify $[Ca^{2+}]_i$ oscillations, changes in $[Ca^{2+}]_i$ were defined as oscillations if their amplitude was equal, to or more than, the half-maximum response observed in 10 mM glucose.

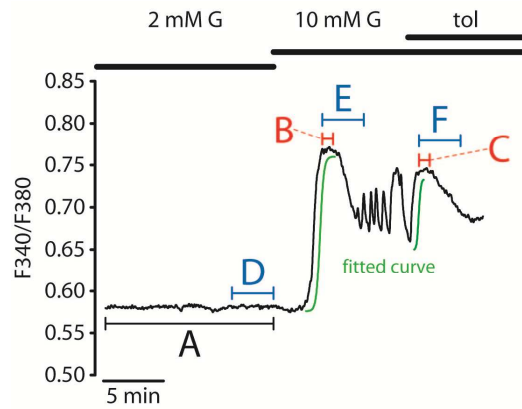


Fig. 5.1 Example of a representative $[Ca^{2+}]_i$ response recorded from a pancreatic islet stimulated with 10 mM glucose and 0.2 mM tolbutamide (tol) plus 10 mM glucose

The parameters analysed are shown. $[Ca^{2+}]_i$ was measured over two different periods in 2 mM glucose (A, D), 10 mM glucose (B, E) and 0.2 mM tolbutamide (tol) plus 10 mM glucose (C, F). **A** Time over which basal $[Ca^{2+}]_i$ in 2 mM glucose was measured. **B-C** Time over which the peak $[Ca^{2+}]_i$ response in 10 mM glucose (B) or 0.2 mM tolbutamide plus 10 mM glucose (C) was measured. Approximately 60 data points, corresponding to a time interval of 30 sec, were averaged (red line). **D-F** Time over which $[Ca^{2+}]_i$ in 2 mM glucose (D), 10 mM glucose (E) or 0.2 mM tolbutamide plus 10 mM glucose (F) was measured. Approximately 420 data points, corresponding to a time interval of 3.5 min, were averaged (blue line). The Ca^{2+} increase upon glucose and tolbutamide stimulation was fitted to the Hill equation 1 (green lines). The fitted curves have been offset relative to the original data for clarity. Parameters for the Hill equation 1 are given in Chapter 2 (General Methods).

5.3 RESULTS

5.3.1 INSULIN SECRETION FROM PERIFUSED E1506K ISLETS

Figure 5.2 shows the insulin secretory responses of either 7-8-week-old (A, B) or 24-week-old (C, D) wild-type, het-E1506K and hom-E1506K islets perifused with 2 mM glucose, 10 mM glucose and 10 mM glucose plus 0.5 mM tolbutamide. Insulin release is either expressed as a percentage of total insulin content (A, C) or as ng/islet/min (B, D). In both age groups, wild-type islets displayed low basal insulin secretion at 2 mM glucose (Fig. 5.2 A-D). Application of glucose (10 mM) stimulated a rapid increase in insulin secretion from wild-type islets that showed an initial peak (first-phase secretion), followed by a much smaller sustained secretion (second-phase release). The magnitude of the sustained secretion was greater in 24-week-old wild-type islets (Fig. 5.2 C, D) than in 7-8-week-old islets (Fig. 5.2 A, B). The K_{ATP} channel inhibitor tolbutamide produced a further increase in insulin secretion from wild-type islets when added to 10 mM glucose solution.

At 7-8-weeks there was no significant difference in basal insulin secretion (2 mM glucose) between wild-type and het-E1506K islets (Fig. 5.2 A, B). Stimulation with 10 mM glucose elicited an increased first-phase and second-phase insulin secretion that was markedly greater in het-E1506K islets than in wild-type islets. However, in contrast to wild-type islets, tolbutamide had only a marginal effect on het-E1506K islets. Hom-E1506K islets exhibited a significant increase in basal insulin secretion compared to both wild-type and het-E1506K islets (Fig. 5.2 A, B). This is expected since the E1506K mutation closes the K_{ATP} channel, causing the β -cell to secrete insulin independently of glucose metabolism (Huopio, Reimann et al. 2000). Both first- and second-phase insulin secretion were increased in hom-E1506K islets relative to wild-type islets, and were not significantly different from het-E1506K islets.

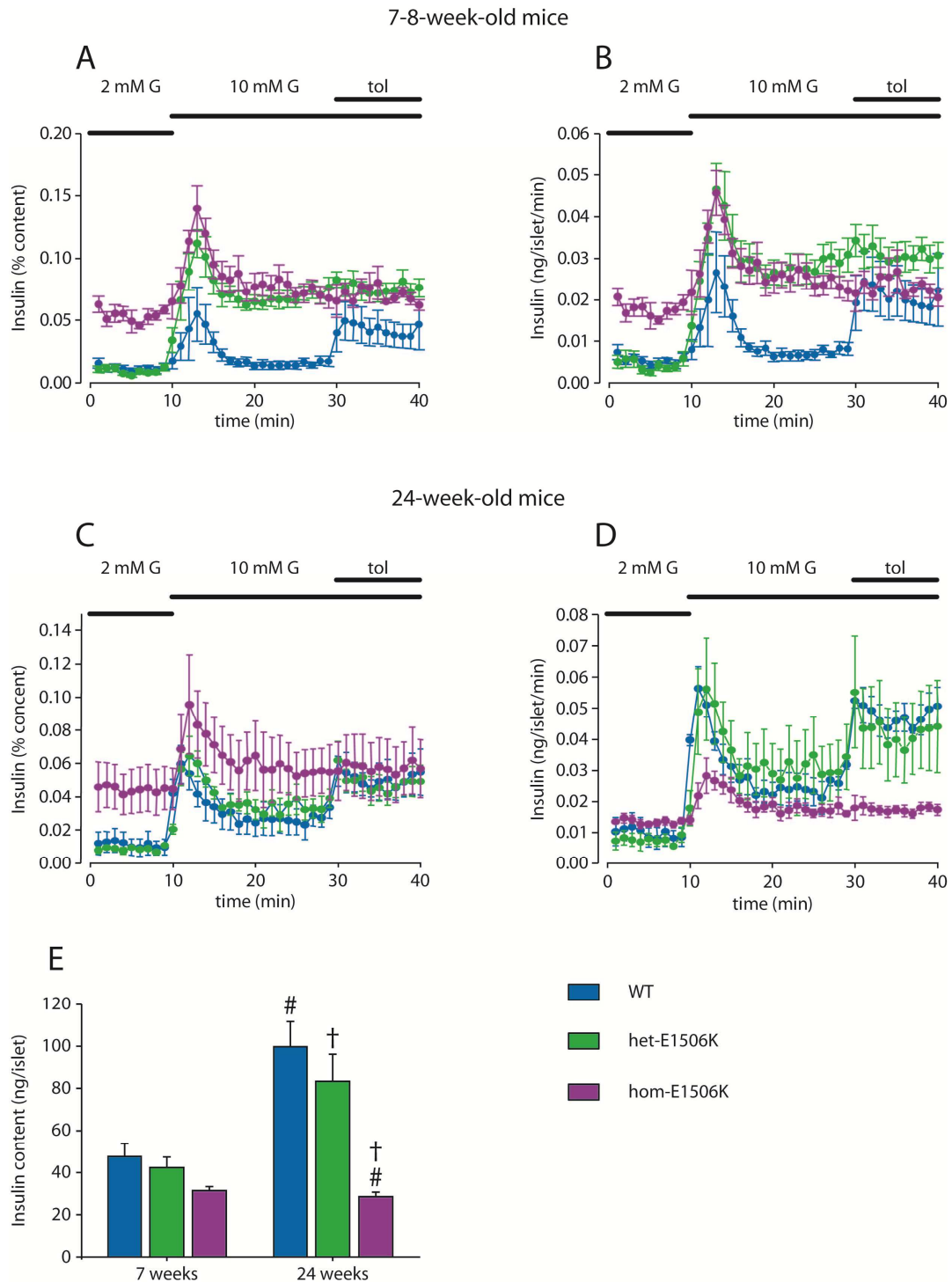


Fig. 5.2 Insulin secretion and insulin content from perfused wild-type, het-E1506K and hom-E1506K islets

Basal insulin secretion was measured at 2 mM glucose. Islets were then perfused with 10 mM glucose followed by 10 mM glucose plus 0.5 mM tolbutamide. **A-B** Insulin secretion from islets isolated from 7-8-week-old mice. Data were expressed as a percentage of insulin content (A) or as ng/islet/min (B). **C-D** Insulin secretion from islets isolated from 24-week-old mice. Data were expressed as a percentage of insulin content (C) or as ng/islet/min (D). **E** Insulin content measured from 7- or 24-week-old islets. Data were obtained from islets isolated from 4 wild-type mice, 5 het-E1506K mice and 5 hom-E1506K mice. Significance was tested using a One-Way ANOVA test and the Bonferroni post-test. Means with the same symbols are significantly different. #, † $p < 0.05$. Data by courtesy of Dr Kenju Shimomura.

Tolbutamide failed to further increase insulin secretion from hom-E1506K islets (as expected if K_{ATP} channels are already closed).

At 24 weeks het-E1506K islets displayed similar insulin secretory responses to wild-type islets (Fig. 5.2 C, D): there were no differences in basal, first- or second-phase secretion. Tolbutamide produced a further increase in insulin secretion in both group of islets.

In 24-week-old hom-E1506K islets, basal insulin secretion was markedly elevated in comparison to both wild-type and het-E1506K islets when secretion was normalised to content (Fig. 5.2 C). As in 7-8-week-old islets, tolbutamide had no effect on insulin secretion in 24-week-old hom-E1506K islets. Interestingly, when insulin secretion was expressed as ng/islet/min, basal secretion was reduced in hom-E1506K islets to similar levels to wild-type and het-E1506K islets (Fig. 5.2 D). This can be explained by the fact that there was a significant decrease in the insulin content of hom-E1506K islets, compared with wild-type or het-E1506K islets, at 24 weeks of age (Fig. 5.2 E).

5.3.2 MEASUREMENTS OF $[Ca^{2+}]_i$ IN 5-6-WEEK-OLD E1506K ISLETS

In the study of Huopio et al., patients heterozygous for the SUR1-E1506K mutation display HI at puberty or early adolescence (Huopio, Reimann et al. 2000). It is therefore reasonable to assume that mice show a comparable phenotype when they reach sexual maturity at 6-8 weeks. Thus, $[Ca^{2+}]_i$ responses to glucose or tolbutamide stimulation were measured in islets isolated from 5-6-week-old wild-type and mutant islets (Fig. 5.3).

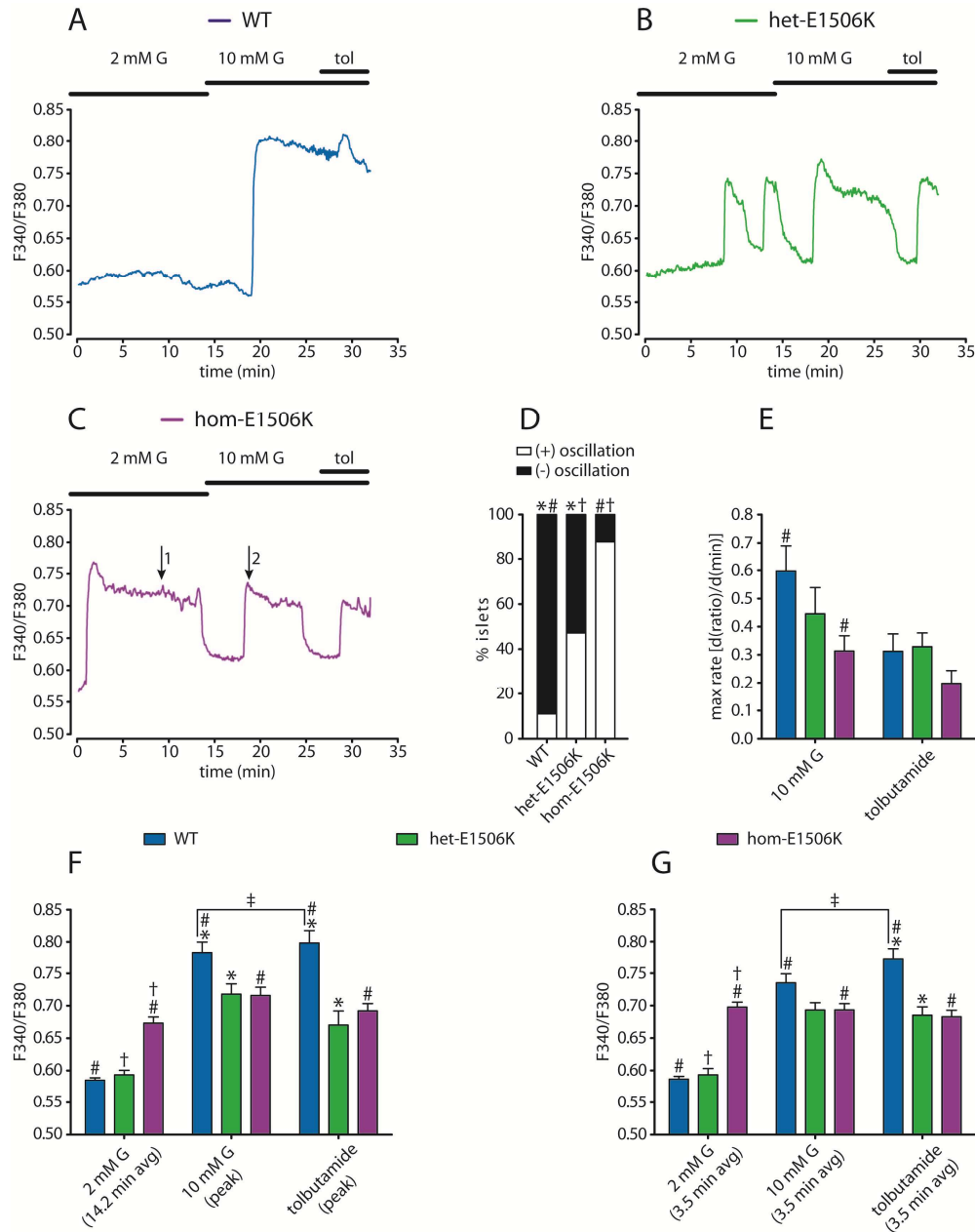


Fig. 5.3 Changes in $[Ca^{2+}]_i$ of 5-6-week-old wild-type and E1506K islets upon stimulation with 10 mM glucose and 0.2 mM tolbutamide

A-C Representative $[Ca^{2+}]_i$ responses recorded from wild-type (A), het-E1506K (B) and hom-E1506K (C) islets. **D** Percentage of islets showing spontaneous Ca^{2+} oscillations in 2 mM glucose (WT = 2/18, het-E1506K = 8/17, hom-E1506K = 21/25). Significance was verified by the Fisher's exact test. **E** Mean ($\pm$ SEM) maximum rate of change in $[Ca^{2+}]_i$ produced by 10 mM glucose or 0.2 mM tolbutamide plus 10 mM glucose. WT (18 islets, tolbutamide), het-E1506K (11 islets, tolbutamide) and hom-E1506K (12 islets, tolbutamide). **F** Mean data ($\pm$ SEM) showing the average Ca^{2+} level in 2 mM glucose, the peak value in 10 mM glucose and the peak value in 0.2 mM tolbutamide plus 10 mM glucose. **G** Mean data ($\pm$ SEM) showing $[Ca^{2+}]_i$ responses averaged over 3.5 min in 2 mM glucose, 10 mM glucose and 0.2 mM tolbutamide plus 10 mM glucose. If not stated otherwise, data are the mean $\pm$ SEM of 18 islets isolated from 4 WT mice, 17 islets isolated from 3 het-E1506K mice and 25 islets from 3 hom-E1506K mice. Significance was tested using a One-Way ANOVA test and the Bonferroni post-test. Compared groups: WT with het-E1506K (*), WT with hom-E1506K (#) and het-E1506K with hom-E1506K (†). As islets were stimulated consecutively with glucose and tolbutamide, the paired t-test (‡) was used to compare the data between different stimuli. *, #, †, ‡ $p < 0.05$.

Wild-type islets exhibited a stable level of basal $[Ca^{2+}]_i$ in 2 mM glucose solution (Fig. 5.3 A), whereas both het-E1506K and hom-E1506K islets displayed spontaneous Ca^{2+} oscillations (Fig. 5.3 B-C). Fig. 5.3 D shows that the percentage of het-E1506K and hom-E1506K islets exhibiting oscillations was significantly different from wild-type islets ($P < 0.0001$ between wild-type and hom-E1506K islets and $P = 0.03$ between wild-type and het-E1506K islets).

In hom-E1506K islets, oscillations occurred in about 80 % of the islets; the remaining 20 % of islets exhibited a stable but elevated level of $[Ca^{2+}]_i$. As a consequence, when $[Ca^{2+}]_i$ was averaged over the full period of exposure to 2 mM glucose, or over the final 3.5 min, it was significantly elevated in hom-E1506K islets (Fig. 5.3 F). Het-E1506K islets showed spontaneous oscillations at 2 mM glucose in 50 % of the islets and the remaining islets showed a stable, low level of $[Ca^{2+}]_i$ comparable of that to wild-type islets. The average $[Ca^{2+}]_i$, measured over the full period of exposure to 2 mM glucose or over the final 3.5 min of exposure (Fig. 5.3 F, G), was not significantly different from wild-type islets.

The maximum rate of rise of the Ca^{2+} signal in response to stimulation with glucose and tolbutamide is shown in Fig. 5.3 E. The maximum rate of change in $[Ca^{2+}]_i$ upon 10 mM glucose stimulation, was reduced approximately two-fold in hom-E1506K islets, when compared with wild-type. Tolbutamide elicited a similar maximum rate of $[Ca^{2+}]_i$ increase in all genotypes.

When glucose was increased to 10 mM, wild-type islets showed a marked increase in $[Ca^{2+}]_i$ (Fig. 5.3 A). The subsequent addition of the K_{ATP} channel blocker tolbutamide (0.2 mM) produced a further small increase in $[Ca^{2+}]_i$ in some, but not in all, cells. The mean Ca^{2+} peak responses to 10 mM glucose or to 0.2 mM tolbutamide plus 10 mM glucose are shown in figure 5.3 F: there was a small (2%) but significant increase ($P = 0.04$) when tolbutamide was added to the glucose solution. Figure 5.3 G reveals a similar increase (5%) after tolbutamide application when $[Ca^{2+}]_i$ was measured as the average $[Ca^{2+}]_i$ over 3.5 min ($P = 0.008$).

Both mutant genotypes exhibited a reduction in the peak $[Ca^{2+}]_i$ response to both 10 mM glucose or to tolbutamide plus 10 mM glucose when compared to wild-type islets (Fig. 5.3 F). These stimuli also produced a smaller increase in $[Ca^{2+}]_i$, when $[Ca^{2+}]_i$ was averaged over 3.5 min (Fig. 5.3 G). In hom-E1506K islets that exhibited elevated $[Ca^{2+}]_i$ in 2 mM glucose (Fig. 5.3 C, arrow 1), addition of 10 mM glucose caused a marked initial decrease in $[Ca^{2+}]_i$, which was then followed by an increase to a level similar to or higher than that observed in 2 mM glucose (Fig. 5.3 C, arrow 2). Consequently hom-E1506K islets did not show any difference in the mean $[Ca^{2+}]_i$ response between 2 mM glucose and 10 mM glucose (Fig. 5.3 G). Neither het-E1506K nor hom-E1506K islets showed a further increase in $[Ca^{2+}]_i$ upon stimulation with tolbutamide plus 10 mM glucose (Fig. 5.3 F, G). This can be explained if their K_{ATP} channels are fully closed in 10 mM glucose.

5.3.3 STIMULATION OF 5-WEEK-OLD HET-E1506K ISLETS WITH TOLBUTAMIDE AND KCL

I next tested the effects of tolbutamide and high potassium on $[Ca^{2+}]_i$ in het-E1506K islets at a non-stimulatory glucose concentration (2 mM). Hom-E1506K islets were not tested as they always exhibit a high basal $[Ca^{2+}]_i$.

Islets were first exposed to 2 mM glucose alone and then sequentially stimulated with 0.2 mM tolbutamide or 70 mM KCl (Fig. 5.4 A, B). After each stimulation the Ca^{2+} transient was allowed to return to basal levels. This recovery showed a hump in both wild-type and het-E1506K islets and can be ascribed to a slow release of Ca^{2+} from the endoplasmic reticulum (Gilon, Arredouani et al. 1999; Lemmens, Larsson et al. 2001). Tolbutamide stimulation elicited a significantly smaller $[Ca^{2+}]_i$ response in het-E1506K islets than in wild-type islets (Fig. 5.4 C).

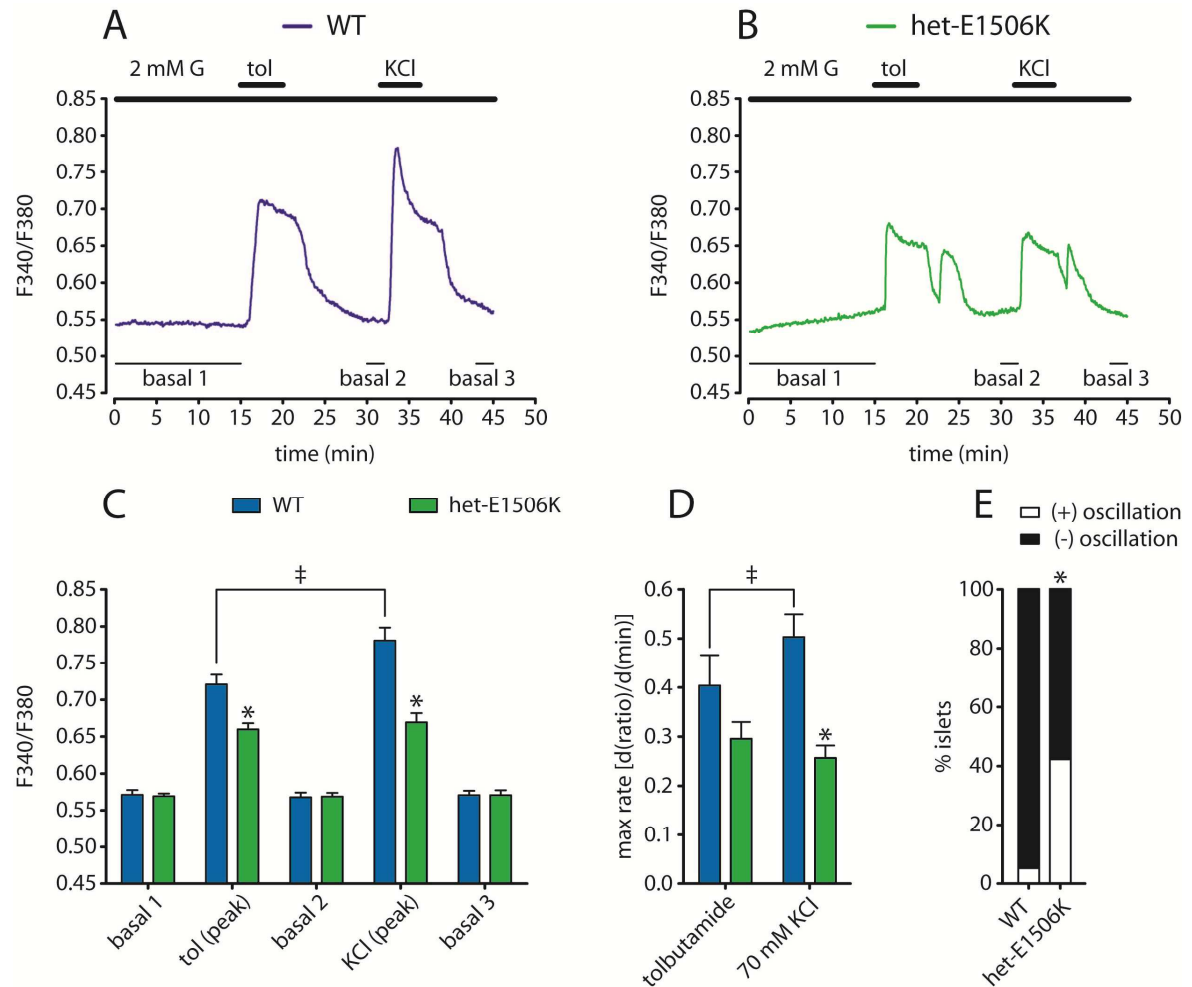


Fig. 5.4 Changes in $[Ca^{2+}]_i$ recorded from 5-week-old wild-type or het-E1506K islets upon stimulation with 0.2 mM tolbutamide and 70 mM KCl

A-B Representative Ca^{2+} signals recorded from wild-type (A) and het-E1506K (B) islets. Glucose (2 mM), tolbutamide (0.2 mM) and KCl (70 mM) were applied as indicated. **C** Mean response ($\pm$ SEM) of wild-type and het-E1506K islets to 0.2 mM tolbutamide or 70 mM KCl. Data were averaged for the period indicated by the horizontal bars below the trace. **D** Mean data ($\pm$ SEM) of the maximum rate of change in $[Ca^{2+}]_i$. **E** Percentage of islets showing spontaneous Ca^{2+} oscillations following return to 2 mM glucose (WT = 1/20, het-E1506K = 10/24). The Fisher's exact test was used to verify significance. Data were expressed as mean and SEM of 21 WT islets (3 mice) and 24 het-E1506K islets (3 mice). As islets were stimulated consecutively with tolbutamide and KCl, the paired t-test was used to compare the data between different stimuli. $\ddagger$ $p < 0.05$. * $p < 0.05$ for unpaired t-test (C, D) and Fisher's exact test (E).

KCl triggers the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels independently of K_{ATP} channels by depolarising the plasma membrane. Assuming a cytoplasmic potassium concentration of 140 mM (Aidley and Stanfield 1996), addition of 70 mM potassium to the extracellular solution would shift the equilibrium potential of potassium to about -18 mV. This

depolarisation exceeds the threshold potential (~ -50 mV) at which electrical activity is initiated (Ashcroft and Rorsman 1989). Consequently, action potentials are triggered, causing an influx of Ca^{2+} into the cell.

In wild-type islets the KCl-induced increase in $[\text{Ca}^{2+}]_i$ was greater than that produced by tolbutamide, suggesting that 70 mM KCl resulted into a stronger depolarisation than that produced by tolbutamide. In het-E1506K islets stimulation with 70 mM KCl produced no further increase in $[\text{Ca}^{2+}]_i$ in comparison to the tolbutamide stimulus. Interestingly, for both tolbutamide and KCl, the magnitude of the Ca^{2+} transient was lower in het-E1506K than in wild-type islets. Furthermore, the maximum rate of rise of Ca^{2+} signal increase was found to be reduced for both the tolbutamide and the KCl stimulus in het-E1506K islets (Fig. 5.4 D). It was also noticeable that het-E1506K islets often showed spontaneous Ca^{2+} oscillations when recovering from stimulation by glucose or KCl (Fig. 5.4 E). The percentage of islets displaying oscillations was significantly different between wild-type and het-E1506K islets ($P = 0.005$).

5.3.4 MEASUREMENTS OF $[\text{Ca}^{2+}]_i$ IN 7-14-MONTH-OLD E1506K ISLETS

It has been reported that mothers of SUR1-E1506K patients show glucose intolerance during pregnancy and go on to develop diabetes later in life (Huopio, Reimann et al. 2000; Vieira, Bergamin et al. 2009). Additionally, their grandmothers often displayed diabetes. Although the exact age at which the affected parents and grandparents developed diabetes was not stated in these papers it is probably safe to assume that the onset of diabetes is likely to occur in the second third of their life. Extrapolating this to the 2 - 2.5 year lifespan of C57BL/6 mouse (Kohn 1971), diabetes is expected to approximately start at about 8 months. It was therefore hypothesised that both het-E1506K and hom-E1506K islets might show decreased glucose-stimulated $[\text{Ca}^{2+}]_i$ responses at that age.

Resting Ca^{2+} levels were similar in het-E1506K and wild-type islets isolated from 7-14-month-old mice (Fig. 5.5 A, B, F, G). Spontaneous Ca^{2+} oscillations in 2 mM glucose were less frequent and not significantly different to wild-type islets (Fig. 5.5 D). Spontaneous Ca^{2+} oscillations were also absent in hom-E1506K islets which only showed continuously elevated Ca^{2+} levels at 2 mM glucose. Both hom-E1506K and het-E1506K islets showed smaller increases in both peak $[\text{Ca}^{2+}]_i$ and average $[\text{Ca}^{2+}]_i$ over 3.5 min than wild-type islets, in response to either 10 mM glucose or 0.2 mM tolbutamide plus 10 mM glucose (Fig. 5.5 A-C, F, G).

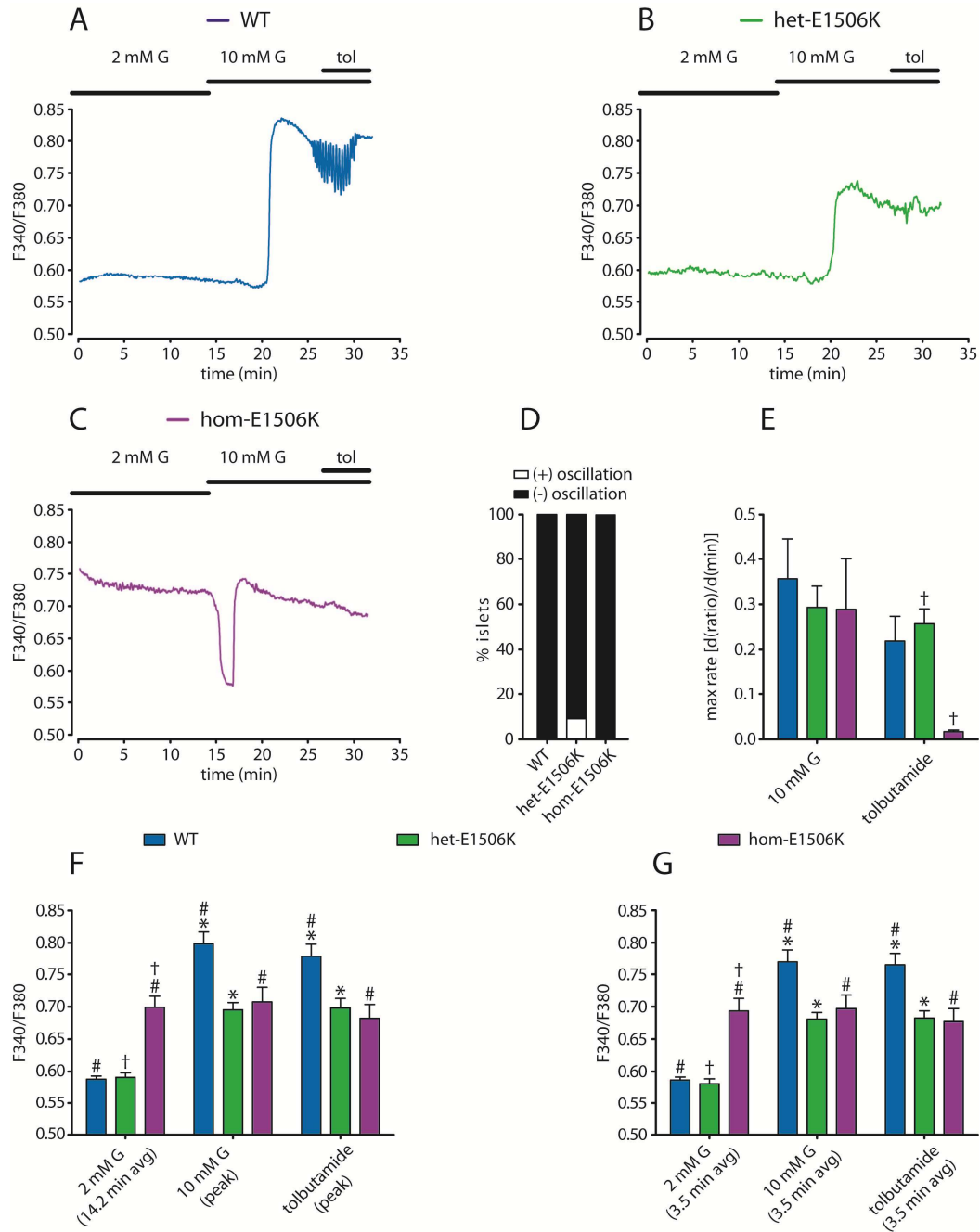


Fig. 5.5 Changes in $[Ca^{2+}]_i$ of 7-14-month-old wild-type and E1506K islets upon stimulation with 10 mM glucose and 0.2 mM tolbutamide plus 10 mM glucose

A-C Representative $[Ca^{2+}]_i$ responses recorded from wild-type (A), het-E1506K (B) and hom-E1506K (C) islets. **D** Percentage of islets showing spontaneous Ca^{2+} oscillations in 2 mM glucose (WT = 0/11, het-E1506K = 4/22, hom-E1506K = 0/11). Significance was analysed with the Chi-square test. No significance was reported. **E** Mean ($\pm$ SEM) maximum rate of change in $[Ca^{2+}]_i$ produced by 10 mM glucose or tolbutamide plus 10 mM glucose, with hom-E1506K ($n = 3$ islets, tolbutamide). **F** Mean data ($\pm$ SEM) showing the average Ca^{2+} level at 2 mM glucose, the peak value at 10 mM glucose and the peak value at tolbutamide plus 10 mM glucose. **G** Mean data ($\pm$ SEM) showing $[Ca^{2+}]_i$ responses averaged over 3.5 min at 2 mM glucose, 10 mM glucose and tolbutamide plus 10 mM glucose. If not stated otherwise, data are the mean $\pm$ SEM of 11 islets (2 WT mice), 22 islets (5 het-E1506K mice) and 11 islets (2 hom-E1506K mice). Significance was tested using a One-Way ANOVA test and the Bonferroni post-test. Compared groups: WT with het-E1506K (*), WT with hom-E1506K (#) and het-E1506K with hom-E1506K (+). *, #, +, $p < 0.05$.

There was no difference in the rates of maximum Ca^{2+} increase in response to glucose stimulation between the three genotypes. The maximum Ca^{2+} increase rate was significantly lower in stimulated with tolbutamide hom-E1506K islets (Fig. 5.5 E).

5.3.5 CALCIUM RESPONSES OF INDIVIDUAL β -CELLS IN 8-15-WEEK-OLD E1506K ISLETS

Next I performed Ca^{2+} imaging using confocal microscopy. This enables Ca^{2+} signals within individual β -cells of the islets to be measured simultaneously and thus spatial differences in the $[\text{Ca}^{2+}]_i$ response across the whole islet to be measured. β -cells are coupled electrically to each other (Zhang, Galvanovskis et al. 2008) and upon 10 mM glucose stimulation wild-type islets responded with synchronised Ca^{2+} oscillations (Fig. 5.6 A). Interestingly, single β -cells in het-E1506K islets exhibited Ca^{2+} oscillations when exposed to 2 mM glucose solution (Fig. 5.6 A). These oscillations of single β -cells were observed in 65% of all het-E1506K islets imaged. In islets that showed oscillations in 2 mM glucose, only ~26% of β -cells were oscillating. This low percentage might explain why the oscillations were not resolved at the whole islet level. It is noticeable that although single β -cells did not oscillate in synchrony in 2 mM glucose solution, they started to oscillate in-phase with other β -cells on stimulation with 10 mM glucose, and their oscillations remain synchronised throughout stimulation with either 0.2 mM tolbutamide plus 10 mM glucose or 70 mM KCl plus 10 mM glucose.

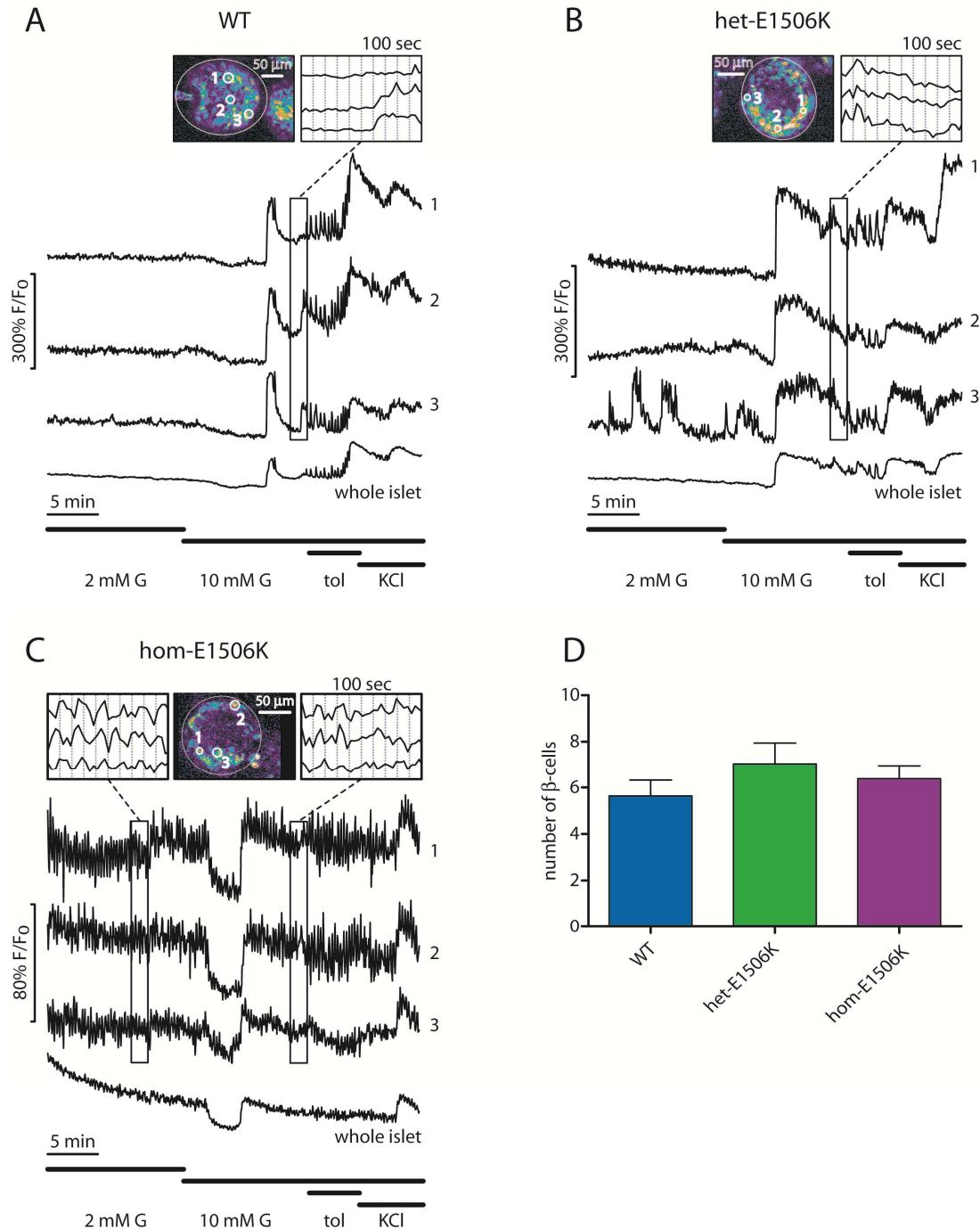


Fig. 5.6 $[Ca^{2+}]_i$ responses in individual β -cells within 8-15-week-old wild-type, het-E1506K and hom-E1506K islets

Islets were stimulated consecutively with 10 mM glucose, 0.2 mM tolbutamide plus 10 mM glucose and 70 mM KCl plus 10 mM glucose. **A-C** Representative Ca^{2+} signals recorded simultaneously from three individual β -cells (as indicated) and the average response of the whole islet (lowest trace) for wild-type (A), het-E1506K (B) and hom-E1506K (C) islets. In the upper panel, the three β -cells are shown on images of the islets. Insets (top) show amplified sections of Ca^{2+} oscillations. **D** Number of β -cells per confocal plane (20 μ m) in wild-type, het-E1506K and hom-E1506K islets responding to 10 mM glucose. The glucose stimulation lasted for 20 min. Significance was tested using a One-Way ANOVA test. No significance was detected. Data were expressed as mean ($\pm$ SEM) of 22 WT islets (3 mice), 23 het-E1506K islets (2 mice) and 32 hom-E1506K islets (3 mice).

Hom-E1506K islets displayed elevated Ca^{2+} levels in 2 mM glucose, with vigorous and synchronised oscillations. After an initial lowering of $[\text{Ca}^{2+}]_i$ caused by the addition of 10 mM glucose, $[\text{Ca}^{2+}]_i$ rose rapidly and oscillations were resumed. This finding is consistent with measurements of $[\text{Ca}^{2+}]_i$ on whole islets of 5-week-old hom-E1506K islets. To see whether the number of β -cells participating in the response to glucose stimulation differed between the heterozygous and homozygous state, the number of β -cells in the confocal plane was counted (Fig. 5.6 D). It turned out that there was no difference in the number of glucose-responsive β -cells between the three different genotypes.

5.3.6 VOLTAGE-GATED INWARD CURRENTS OF SUPERFICIAL α - AND β -CELLS IN HET-E1506K ISLETS OF 5-6-WEEK-OLD MICE

Because depolarisation with tolbutamide or KCl produced a smaller rise in $[\text{Ca}^{2+}]_i$ in het-E1506K islets than in wild-type islets, I investigated whether this decrease in $[\text{Ca}^{2+}]_i$ could be attributed to a reduction of Ca^{2+} influx through voltage-dependent Ca^{2+} channels in β -cells. Pancreatic islets contain both α -cells (15 %) and β -cells (80 %) (MacDonald and Rorsman 2006). In the following experiments I measured the voltage-activated inward current in both of these cell types by patch-clamping individual cells in intact islets. Individual β - and α -cells were identified by the different inactivation properties of their Na^+ channels (Fig. 5.7). Na^+ currents were elicited by a two-pulse inactivation protocol: a 50 ms long, conditioning pre-pulse to voltages between -180 and -5 mV was used to inactivate the Na^+ channels. This was followed by a test pulse to $+10$ mV.

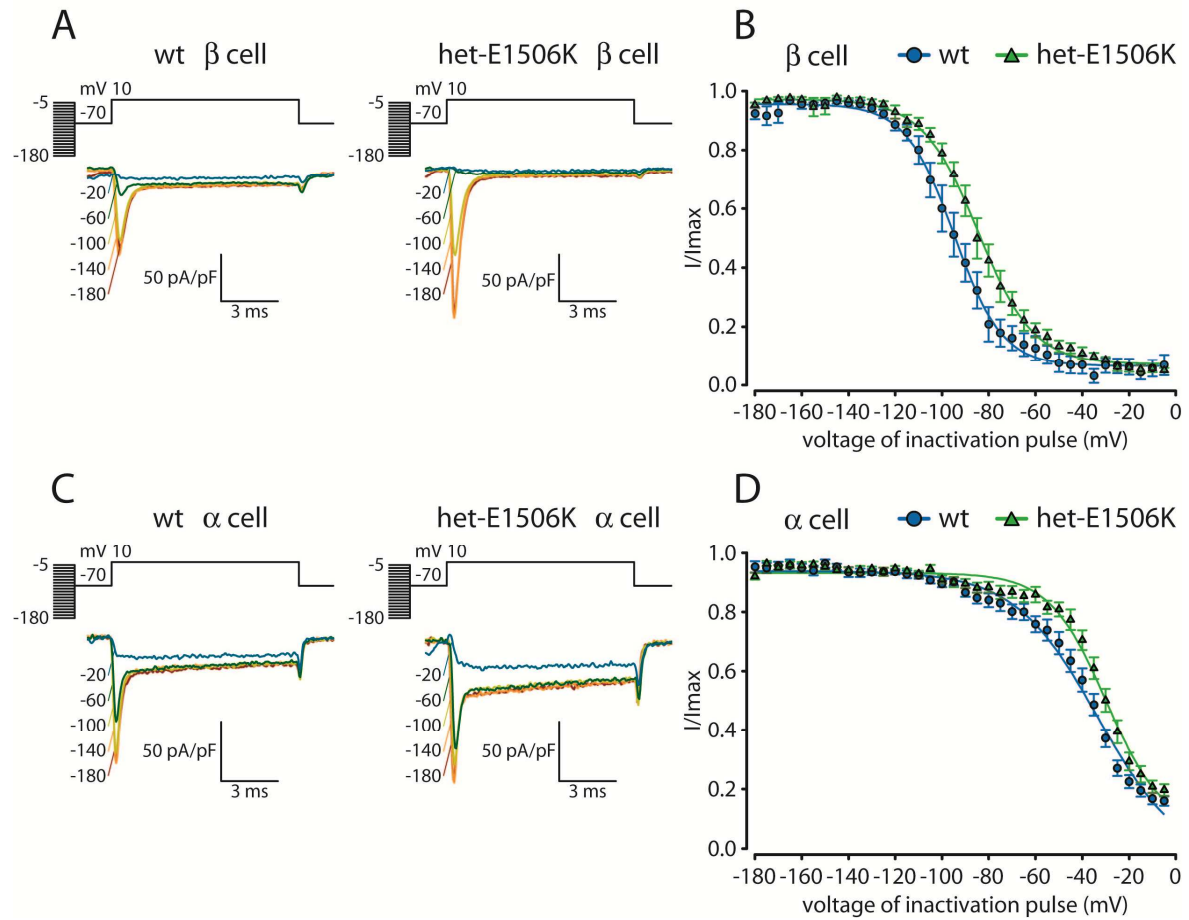


Fig. 5.7 Identification of α - and β -cells in intact wild-type and het-E1506K islets by the inactivation properties of their inward currents

A, C Series of representative inward currents from wild-type (WT) or mutant (het-E1506K) β -cells (A) and α -cells (C). Currents were elicited by a two-pulse inactivation protocol (Na^+ steady-state inactivation h_{∞} protocol). The first 50 ms long pulse was varied between -180 and -5 mV. The second pulse was always to +10 mV. The top trace indicates the voltage protocol, the lower trace is the recorded current. Only raw data from the indicated voltages are shown for clarity. **B, D** Relationship between the conditioning voltage (pre-pulse) and the normalised current amplitude ($I/I_{\max}$) of the second pulse measured for β -cells (B) and α -cells (D). The curves were obtained by fitting the Boltzmann equation to the data using the parameters given in the text. Data were collected from 2 WT and 2 het-E1506K mice, each 5-6 weeks old. Data points were expressed as mean $\pm$ SEM of 12 WT β -cells (for one β -cell per islet) and 13 mutant β -cells as well as 8 WT α -cells and 10 mutant α -cells.

The Na^+ current inactivation curve was obtained by fitting the Boltzmann equation (equation 5.1) to the data points:

$$h_{\infty}(Vm) = h_{max} + \frac{h_{min} - h_{max}}{1 + e^{\frac{V_h - V_m}{k_h}}} \quad (5.1)$$

where h_{∞} is the current amplitude during the test pulse following an inactivating prepulse normalised to the maximum current in the absence of the prepulse (h_{max}). V_m is the membrane potential of the pre-pulse and V_h is the membrane potential at which h_{∞} is halfway between the maximum current (h_{max}) and the minimum current (h_{min}). k_h is the steepness of the curve.

The β -cell Na^+ channel is half-inactivated at a membrane potential of -104 mV and is fully inactivated at around -80 mV (Plant 1988; Gopel, Kanno et al. 1999). However, in α -cells, V_h is reported to be -47 mV (Gopel, Kanno et al. 2000). Therefore, I measured inactivation curves for each cell and defined β -cells as those having an inward current that showed half-maximal activation (V_h) between -114 mV and -71 mV. I defined α -cells as those where V_h lay between -49 mV and -17 mV.

There was no difference in the V_h of the inward current (means of the individual fits) recorded from α -cells from wild-type (-34 ± 3 mV) and het-E1506K islets (-29 ± 2 mV), as shown in figure 5.7 D. However, β -cells from het-E1506K islets displayed a significant shift in V_h of $+10$ mV ($P = 0.03$): the means of the individually fits of V_h values was 93 ± 3 mV for wild-type islets and 83 ± 3 mV for het-E1506K islets (Fig. 5.7 B).

Next, voltage-clamp depolarisations were applied in 12 ms long pulses to activate voltage-gated inward currents from a holding potential of -70 mV which should be positive enough to inactivate most of the Na^+ current in β -cells (Fig. 5.8).

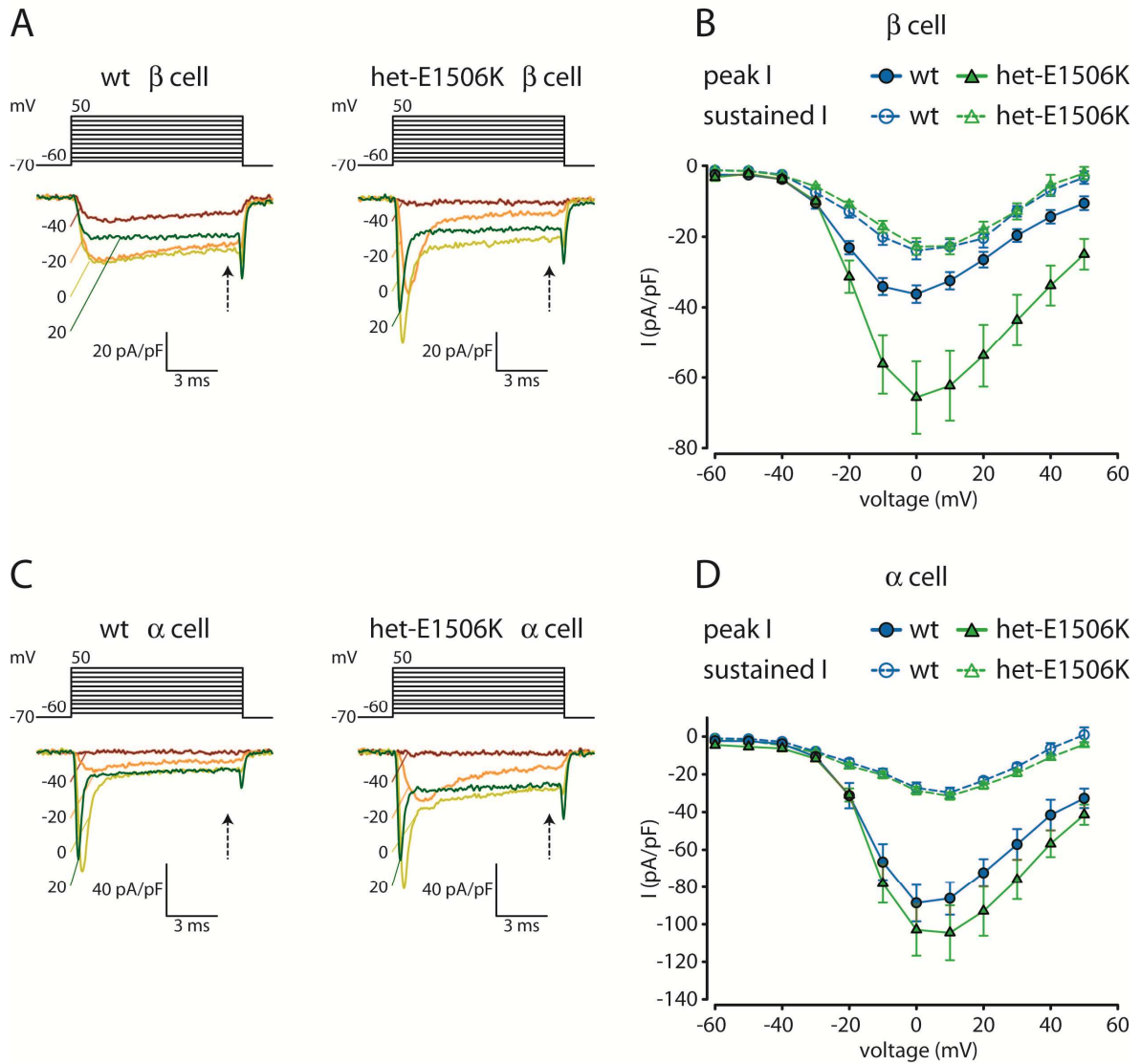


Fig. 5.8 Voltage-activated inward currents recorded from α - and β -cells in intact wild-type and het-E1506K islets

A, C Representative voltage-activated inward currents recorded from wild-type (WT) or mutant (het-E1506K) β -cells (A) and α -cells (C). Currents were evoked by depolarising voltage steps from -60 mV to +50 mV (in 10 mV increments) from a holding potential of -70 mV. Only currents evoked by the indicated voltage steps are shown for clarity. The arrows indicate the time point at which the sustained current was measured. **B, D** Relation between the peak inward current (peak I, solid line) and the cell membrane potential, and sustained current (sustained I, dotted line) and the cell membrane potential in β -cells (B) and α -cells (D). Data points were expressed as mean $\pm$ SEM (for one β -cell per islet) of 12 WT β -cells and 13 mutant β -cells (B), or 8 WT α -cells and 10 mutant α -cells (D).

Potassium currents were blocked by tetraethylammonium (TEA). Both the peak amplitude of the inward current as well as the sustained current measured at the end of the pulse were recorded

during depolarising pulses ranging from -60 and $+50$ mV. Data were expressed as pA per pF to account for differences in cell size.

Surprisingly, het-E1506K β -cells showed a significantly larger voltage-activated inward current with faster activation kinetics than β -cells from wild-type islets (Fig. 5.8 A, B). Their I-V curve diverged at about -20 mV and amounted to a maximum at 0 mV of -36 ± 2 pA/pF for wild-type β -cells and -66 ± 10 pA/pF for het-E1506K β -cells ($P = 0.02$). In α -cells the I-V relationship also displayed a maximum current at 0 mV (Fig. 5.8 D). In contrast to β -cells, voltage-activated currents at 0 mV in α -cells did not show any differences between wild-type (-89 ± 10 pA/pF) and het-E1506K (-103 ± 14 pA/pF) islets.

In both β - and α -cells Na^+ currents are inactivated after about 5 ms (Plant 1988; Gopel, Kanno et al. 1999; Gopel, Kanno et al. 2000). Thus the sustained current measured at the end of the pulse (11 ms after the onset of the pulse as indicated by the arrows in figure 5.8 A, C) could be used to estimate the Ca^{2+} current. As shown in figure 5.8 B, D (dotted line) the magnitude of this current is similar in wild-type and het-E1506K β -cells (B), as well as in wild-type and het-E1506K α -cells (D). This suggests that the peak current recorded from het-E1506K β -cells presumably contains a Na^+ current and that the Ca^{2+} influx has been unaltered in het-E1506K islets.

5.4 DISCUSSION

The experiments carried out in this chapter were performed on mice from different age groups. Although the ages of mice used for $[Ca^{2+}]_i$ measurements (5-6 weeks and 7-14 month) and insulin secretion (7-8 weeks and 6 month) were not identical, I will discuss them as if they were and name them young mice and old mice, respectively. $[Ca^{2+}]_i$ responses of individual β -cells were examined in islets isolated from 8-15-week-old mice. This age constitutes an intermediate age group between young and old mice and will be considered separately.

5.4.1 $[Ca^{2+}]_i$ RESPONSES IN ISLETS ISOLATED FROM YOUNG MICE

Wild-type islets displayed a stable and low Ca^{2+} level in 2 mM glucose. In most islets, 10 mM glucose produced an initial small fall in $[Ca^{2+}]_i$, which is believed to be mediated by the action of sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pumps that remove Ca^{2+} from the cytoplasm into intracellular stores (Chow, Lund et al. 1995; Tengholm, Hellman et al. 2001). The initial decrease in $[Ca^{2+}]_i$ was followed by a robust increase in $[Ca^{2+}]_i$. Application of 0.2 mM tolbutamide plus 10 mM glucose to young wild-type islets produced an even further increase in $[Ca^{2+}]_i$, indicating that tolbutamide was able to further block K_{ATP} channel current.

About 50% of young het-E1506K islets showed spontaneous Ca^{2+} oscillations when exposed to 2mM glucose. These oscillations started from a low $[Ca^{2+}]_i$ and did not result in an mean elevation in average $[Ca^{2+}]_i$. Because the β -cell K_{ATP} channel consists of four SUR1 subunits (Clement, Kunjilwar et al. 1997), het-E1506K cells will express a mixed population of mutant and wild-type K_{ATP} channels. Assuming wild-type and SUR1-E1506K mutant subunits assemble randomly (Shyng and Nichols 1997), the distribution of mutant and wild-type subunits in the heterozygous channel population will look as follows: one-sixteenth of channels will contain 4 mutant subunits, fourteen-sixteenth of channels will contain a mixture of mutant and wild-type

subunits (between one and three) and one-sixteenth of channels will contain 4 wild-type subunits. This binomial distribution seems to be reasonable to assume since the mutant *ABCC8* gene encoding SUR1-E1506K is driven by its endogenous promoter, and will therefore show normal regulation of gene expression. Thus, channel activities may be variable based on the number of mutant subunits present and the extent to which the mutant subunit exerts its adverse effect on channel activity.

Huopio et al. showed that in *Xenopus* oocytes the amplitude of the whole-cell currents through K_{ATP} channels derived from heterologous expression of Kir6.2 and a 1:1 mixture of wild-type and mutant SUR1-E1506K was intermediate between that recorded for the wild-type K_{ATP} channel and the Kir6.2/SUR1-E1506K channel (Huopio, Reimann et al. 2000). This suggests that the SUR1-E1506K mutation does not exert a dominant negative effect on wild-type SUR1.

In young het-E1506K islets, stimulation with 10 mM glucose produced an increase in $[Ca^{2+}]_i$ whose magnitude was significantly smaller than in wild-type islets. Additional stimulation with tolbutamide did not further increase $[Ca^{2+}]_i$ and the mean $[Ca^{2+}]_i$ response was smaller than in wild-type islets. Similarly, depolarisation of young het-E1506K islets with tolbutamide (0.2 mM) or KCl (70 mM) in the presence of 2 mM glucose resulted in a rise in $[Ca^{2+}]_i$ that was slower and smaller than in wild-type islets. This change in $[Ca^{2+}]_i$ can be attributed to either a reduced Ca^{2+} influx through voltage-gated Ca^{2+} channels or an enhanced Ca^{2+} clearance from the cytosol of het-E1506K islets.

The removal of Ca^{2+} from the cytosol is carried out by the action of ATP-powered pumps and transporters: the plasma membrane Na^+/Ca^{2+} exchanger (NCX), plasma membrane Ca^{2+} -ATPase (PMCA), SERCA pumps, and the calcium uniporter of mitochondria (Chen, Koh et al. 2003). In the wild-type pancreatic β -cell most Ca^{2+} is removed by SERCA (Chen, Koh et al. 2003; Dufer, Haspel et al. 2007). However, β -cells lacking K_{ATP} channel, or cells in which these channels were blocked, display altered Ca^{2+} handling by recruiting non-ER Ca^{2+} stores (Dufer, Haspel et al. 2007).

The sequestration of Ca^{2+} into the ER is believed to take place a few seconds after the depolarising stimulus (Gilon, Arredouani et al. 1999; Chen, Koh et al. 2003). Thus, it is possible that an altered Ca^{2+} clearance in het-E1506K islets is responsible for the smaller increase in $[\text{Ca}^{2+}]_i$ upon stimulation with either glucose, tolbutamide or KCl. To test this idea, $[\text{Ca}^{2+}]_i$ responses in het-E1506K islets in the presence of drugs that inhibit the specific pumps and transporters involved in the clearance of Ca^{2+} need to be investigated.

Nearly all young hom-E1506K islets displayed spontaneous Ca^{2+} oscillations in 2 mM glucose: remaining islets showed constantly elevated Ca^{2+} levels. Consequently, the average $[\text{Ca}^{2+}]_i$ in 2 mM glucose was increased. This can be explained by a reduction in K_{ATP} current. Whole-cell currents recorded from recombinant Kir6.2/SUR1-E1506K channels expressed in *Xenopus* oocytes were found to be greatly reduced, because MgADP failed to open the channel on metabolic inhibition (Huopio, Reimann et al. 2000). Thus, at low glucose levels when MgADP levels are high and MgATP low, the SUR1-E1506K K_{ATP} channel remains shut, resulting in permanent membrane depolarization and elevated $[\text{Ca}^{2+}]_i$.

Stimulation with 10 mM glucose induced a transient fall in $[\text{Ca}^{2+}]_i$ in hom-E1506K islets which quickly recovered to initial values observed in 2 mM glucose. This indicates that K_{ATP} channels were already completely closed, leading to no further depolarisation or Ca^{2+} influx upon stimulation with 10 mM glucose. The glucose-stimulated Ca^{2+} transient in hom-E1506K islets is reminiscent of that in islets lacking K_{ATP} channels because of ablation of either SUR1 or Kir6.2, or of wild-type islets in which the K_{ATP} channel was blocked by sulphonylureas (Miki, Nagashima et al. 1998; Shiota, Larsson et al. 2002; Li, Buettger et al. 2004; Nenquin, Szollosi et al. 2004; Szollosi, Nenquin et al. 2007; Ravier, Nenquin et al. 2009). In those islets the lowering of $[\text{Ca}^{2+}]_i$ is believed to be independent of SERCA pumps and Ca^{2+} extrusion may occur via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Dufer, Haspel et al. 2009) as discussed in detail in Chapter 4. It is possible that same molecular

mechanisms that underlie the glucose-stimulated Ca^{2+} transients in K_{ATP} knockout islets, or wild-type islets in which the K_{ATP} channel was blocked, are also operational in hom-E1506K islets.

Although tolbutamide blocks wild-type and SUR1-E1506K K_{ATP} channels to similar degree (Huopio, Reimann et al. 2000), addition of tolbutamide the 10 mM glucose solution did not produce a further increase in $[\text{Ca}^{2+}]_i$. This suggests that the K_{ATP} channel in hom-E1506K islets is already closed. The magnitude of the glucose- and tolbutamide-stimulated $[\text{Ca}^{2+}]_i$ was less than that of wild-type islets but similar to that of het-E1506K islets. It is therefore possible that similar mechanisms, as discussed above, account for the reduced $[\text{Ca}^{2+}]_i$ response in both het-E1506K and hom-E1506K islets.

5.4.2 $[\text{Ca}^{2+}]_i$ RESPONSES IN ISLETS ISOLATED FROM OLD MICE

Islets isolated from old wild-type, het-E1506K or hom-E1506K mice showed no major differences in the magnitude of their $[\text{Ca}^{2+}]_i$ responses to 2 mM glucose, 10 mM glucose or 0.2 mM tolbutamide plus 10 mM glucose in comparison with islets from young mice. However, the spontaneous Ca^{2+} oscillations found in many young het-E1506K or hom-E1506K mice were no longer be observed in old islets. The reason for this is not clear.

5.4.3 $[\text{Ca}^{2+}]_i$ RESPONSES OF INDIVIDUAL B-CELLS IN INTACT ISLETS ISOLATED FROM MICE OF AN INTERMEDIATE AGE GROUP

Wild-type β -cells displayed a stable and low $[\text{Ca}^{2+}]_i$ in 2 mM glucose. Application of 10 mM glucose evoked a robust, simultaneous elevation of $[\text{Ca}^{2+}]_i$, followed by oscillations which were synchronised throughout the β -cell population (Nadal, Quesada et al. 1999). This is believed to be

due to electrical and / or chemical coupling between β -cells within the islet (Santos, Rosario et al. 1991; Zhang, Galvanovskis et al. 2008; Tengholm and Gylfe 2009).

In the majority of het-E1506K islets, about one-fourth of β -cells exhibited spontaneous Ca^{2+} oscillations in 2 mM glucose. This is consistent with studies on islets isolated from heterozygous Kir6.2 knockout mice (Remedi, Rocheleau et al. 2006). In that paper it was shown that heterozygous Kir6.2 knockout mice have a significant (~60%) reduction of K_{ATP} channel activity in their islets. Consequently, when islets were incubated with 6 mM glucose the fraction of active β -cells was increased in heterozygous Kir6.2 knockout islets compared with wild-type islets. It is noteworthy that oscillations of β -cells in het-E1506K islets in 2 mM glucose remained isolated and did not affect the rest of the β -cell population. This suggests that current that flows via gap junctional coupling (Speier, Gjinovci et al. 2007) is not strong enough to depolarise neighbouring β -cells in which K_{ATP} channels are not strongly inhibited.

Upon stimulation with 10 mM glucose all β -cells, including those showing Ca^{2+} oscillations in 2 mM glucose, responded simultaneously with an increase in $[\text{Ca}^{2+}]_i$, which was followed by synchronised oscillations amongst β -cells. This observation is consistent with the finding that electrical coupling between islet cells increases with a rise in glucose concentration (Eddlestone, Goncalves et al. 1984).

β -cells in hom-E1506K islets displayed fast synchronous Ca^{2+} oscillations in both 2 mM glucose and 10 mM glucose. These oscillations were reminiscent of oscillations observed in individual β -cells within wild-type islets incubated with glibenclamide (see Chapter 4). Furthermore, studies on clustered β -cells showed that tolbutamide-induced Ca^{2+} oscillations are well synchronised (Jonkers, Guiot et al. 2001). This suggests that closure of K_{ATP} channel, either pharmacologically or genetically, causes synchronous Ca^{2+} oscillations.

5.4.4 INSULIN SECRETION FROM YOUNG ISLETS

Insulin secretion in young wild-type islets evoked by 10 mM glucose exhibited a characteristic biphasic pattern with a rapid but transient increase in secretion (first-phase) followed by a slower and sustained second-phase. Interestingly, second-phase secretion was unusually low in these islets.

Young het-E1506K islets showed no difference in basal secretion compared to wild-type islets. This is consistent with the Ca^{2+} data which revealed no difference of average $[\text{Ca}^{2+}]_i$ in 2 mM glucose between het-E1506K and wild-type islets. Glucose-stimulated first- and second-phase insulin secretion were elevated in het-E1506K islets despite a reduced $[\text{Ca}^{2+}]_i$ response in those islets. This seems to be perplexing. One possibility is that the amplifying pathway in het-E1506K islets is enhanced. Nenquin et al. observed that SUR1 knockout islets displayed an elevated insulin secretion level in comparison to wild-type islets which were incubated with tolbutamide, albeit SUR1 knockout islets showed similar or even lower $[\text{Ca}^{2+}]_i$ (Nenquin, Szollosi et al. 2004). The authors explained this by a modulation of the amplifying pathway, which was suspected to work more effectively in SUR1 knockout islets. This notion was supported by Shiota et al. who showed that sensitivity of the exocytotic machinery to Ca^{2+} was increased in β -cells from SUR1 knockout mice (Shiota, Larsson et al. 2002). Thus, a similar mechanism is conceivable to work in het-E1506K islets. Tolbutamide in combination with 10 mM glucose failed to significantly raise the release of insulin in both het-E1506K islets which agrees with the absence of a $[\text{Ca}^{2+}]_i$ response to the same stimulus.

Islets isolated from young hom-E1506K mice exhibited a significant increase in basal insulin secretion (measured in 2 mM glucose). This is consistent with the elevated $[\text{Ca}^{2+}]_i$ found in hom-E1506K islets in 2 mM glucose. Application of 10 mM glucose stimulated secretion of insulin (first -and second-phase) which was significantly elevated in hom-E1506K islets in respect to wild-type islets and similar to het-E1506K islets. With a similarly reduced glucose-stimulated increase

in $[Ca^{2+}]_i$ it is likely that increased insulin secretion is due to an enhanced amplifying pathway (similar to het-E1506K islets). Tolbutamide plus 10 mM glucose had no effect on average insulin release, which was expected from the failure of the same stimulus to raise $[Ca^{2+}]_i$.

5.4.5 INSULIN SECRETION FROM OLD ISLETS

Old het-E1506K islets showed no difference in basal, first- and second-phase insulin secretion in comparison to wild-type islets. The reason for this age-related change from hypersecretion to normal secretion in het-E1506K islets is unclear. In contrast to young islets, old het-E1506K islets displayed a marked response to tolbutamide. It is possible that this increase in insulin secretion is attributable to the drug acting downstream of the K_{ATP} channel to stimulate exocytosis of insulin (Eliasson, Renstrom et al. 1996). A recent study by Zhang et al. has revealed that sulphonylureas can interact with Epac2, a cAMP-activated guanine nucleotide exchange factors for Ras-like GTPases, and promote insulin secretion (Zhang, Katoh et al. 2009). However why this only happens in old and not in young het-E1506K islets is unknown.

In old hom-E1506K islets, there was no significant difference in basal insulin secretion, when expressed per islet, in comparison to wild-type islets. Furthermore, the amplitude of secretion of insulin upon glucose and tolbutamide stimulation (in ng/islet/min) was significantly decreased in hom-E1506K islets. This reduction in insulin secretion is not due to a defect in the stimulus secretion coupling in hom-E1506K islets, because when secretion is expressed relative to insulin content the secretion rate is still elevated in 2 and 10 mM glucose. The reduction in insulin secretion in old hom-E1506K islets can be explained by a significant decline in insulin content of these islets.

Both old wild-type and old het-E1506K islets had approximately twice the insulin content of young islets. This is consistent with a natural age-related decrease in insulin content in mice

(Leiter, Premdas et al. 1988). In hom-E1506K islets this increase in insulin content was not observed, which may be due to a reduction in β -cell replication, neogenesis or an increase in β -cell death, or a combination of all of these factors (Rhodes 2005).

Ca^{2+} induced apoptosis (Berridge, Lipp et al. 2000) could provide a possible explanation for the reduction in β -cell mass: in HI the β -cell is constantly exposed to depolarisation, increased $[\text{Ca}^{2+}]_i$ and hypersecretion of insulin. Indeed, Efanova et al. showed that long term closure of K_{ATP} channels with glucose or tolbutamide resulted in Ca^{2+} -dependent apoptosis (Efanova, Zaitsev et al. 1998). Conversely, β -cell death could be caused by endoplasmic reticulum stress mediated by the increased production of insulin (Fonseca, Burcin et al. 2009) or ROS formation as discussed in the Chapter 1.

At an intermediate age, the number of glucose-responsive β -cells, determined by confocal Ca^{2+} imaging, was equal in wild-type, het-E1506K and hom-E1506K islets. These data suggest no change in viability of β -cells from different sets of islets. Thus it is likely that the lower insulin content of hom-E1506K islets is due to a reduced insulin production. Such a process was observed in the face of hyperglycemia, when insulin gene expression was found to be suppressed (Harmon, Stein et al. 2005).

5.4.6 ELECTROPHYSIOLOGICAL CHARACTERISATION OF THE HET-E1506K ISLETS

To investigate whether the reduced increase in $[\text{Ca}^{2+}]_i$ in young het-E1506K islets evoked by depolarising stimulation with tolbutamide or KCl was linked to a reduced influx of Ca^{2+} , I measured voltage-activated inward currents in β -cells from intact het-E1506K islets. Unexpectedly, the recorded inward current in het-E1506K β -cells was significant larger than in wild-type cells.

In β -cells, the inward current should exclusively represent Ca^{2+} current, flowing mainly through L-type channels (Keahey, Rajan et al. 1989), because Na^+ channels are known to inactivate completely at the physiological resting potential (about -80 mV) in mouse β -cells (Plant 1988; Gopel, Kanno et al. 1999). The Na^+ steady-state inactivation (h_∞) curve of het-E1506K β -cells, however, appeared to be shifted towards more positive membrane potentials. This would imply that the recorded current in het-E1506K β -cells may be contaminated by Na^+ current, which would add to the Ca^{2+} current and would explain why the inward current was stronger than in wild-type β -cells. In order to investigate the voltage-activated Ca^{2+} current in het-E1506K β -cells it would therefore be necessary to pharmacologically isolate the current by using tetrodotoxin (TTX) to block voltage-gated Na^+ channels.

Alternatively, I measured the sustained current at the end of the depolarisation pulse (11 ms after the onset of the pulse). At this point Na^+ currents are already inactivated in both β - and α -cells (Plant 1988; Gopel, Kanno et al. 1999; Gopel, Kanno et al. 2000). The similar magnitude of the sustained current in wild-type and het-E1506K β -cells indeed suggests that the peak current recorded in het-E1506K β -cells contains a Na^+ current. The data further suggest that the Ca^{2+} influx has not been reduced in het-E1506K β -cells.

Na^+ channels have been found to participate in the generation of action potentials and initiation of insulin secretion in β -cells of species such as human (Barnett, Pressel et al. 1995), rat (Hiriart and Matteson 1988) and dog (Pressel and Misler 1991). In human β -cells, for example, it has been proposed by Braun et al. that insulin secretion is preceded by the following events: glucose metabolism closes K_{ATP} channels, causing depolarisation of the plasma membrane which then leads to the generation of the action potential by activation of different sets of voltage-gated Ca^{2+} channels (T- and L-type Ca^{2+} channels) (Braun, Ramracheya et al. 2008). During the upstroke of the action potential, voltage-gated Na^+ channels open, leading to a stronger

depolarisation of the β -cell and activation of another set of Ca^{2+} channels (P/Q-type Ca^{2+} channels), which culminates in exocytosis of insulin.

It is possible that a shift in the inactivation curve of the Na^+ channels towards more positive membrane potentials results in a contribution of Na^+ channels to the electrical activity of het-E1506K β -cells. This could result in changes of, for example, resting potential, threshold potential or plateau potential. It is likely that this Na^+ current provides a depolarisation step towards the threshold potential which enables the het-E1506K β -cells to fire action potentials at low glucose concentrations (< 7 mM) and may account for the spontaneous Ca^{2+} oscillations observed in β -cells of 5-6-week-old islets at 2 mM glucose.

5.4.7 COMPARISON OF INSULIN SECRETION DATA WITH SUR1-E1506K PATIENTS

Laakso and colleagues have generated a mouse model of HI that expresses a mutation in the SUR1 subunit (E1506K) that causes HI in patients and predisposes them to diabetes later in life (Huopio, Reimann et al. 2000; Pinney, MacMullen et al. 2008; Vieira, Bergamin et al. 2009). Patients suffering from HI were heterozygous for the SUR1-E1506K allele, indicating a dominant mode of inheritance. I will therefore compare the clinical findings with secretion data from het-E2506K islets.

In SUR1-E1506K patients of young age (from birth to puberty), hypoglycemia occurs predominately in episodes suggesting that abnormal insulin release is not constitutive. This is consistent with basal insulin secretion (measured in 2 mM glucose) from het-E2506K islets which was found to not elevated and similar to wild-type islets. Glucose-stimulated first- and second-phase secretion of insulin was elevated in het-E2506K islets and could explain the hypoglycemia of young SUR1-E1506K patients.

The first cases of glucose intolerance in SUR1-E1506K patients were reported for female patients after pregnancy (Huopio, Reimann et al. 2000; Vieira, Bergamin et al. 2009). Insulin secretion was found to decrease linearly with age in SUR1-E1506K patients as glucose tolerance progresses to diabetes. (Huopio, Otonkoski et al. 2003). This is paralleled by a reduction of first- and second-phase glucose-stimulated insulin secretion in old het-E2506K islets compared to young islets. It is likely that the rate of insulin release further declines with age, as there seems to be a trend for old het-E2506K islets to show a reduction in insulin content.

Although the het-E2506K mouse fails to explain the severe symptoms of hypoglycemia in SUR1-E1506K patients which were provoked by fasting, it seems to better model the state of glucose intolerance and diabetes later in life.

5.5 SUMMARY

The SUR1-E1506K mutation of the K_{ATP} channel causes a reduction in channel activity. It is associated with HI in early life and leads to the development of glucose intolerance, insulin deficiency and diabetes later in life.

My studies showed enhanced glucose-stimulated insulin secretion from young het-E1506K (first- and second-phase) and young hom-E1506K islets (basal and first-and second-phase). This increase in glucose-stimulated secretion in both types of islets may be attributed to the amplifying pathway. In old het-E1506K islets glucose-stimulated insulin secretion declined to normal levels, whereas in old hom-E1506K islets basal insulin secretion dropped to normal levels and glucose-stimulated insulin secretion was found to be markedly reduced in comparison to wild-type islets. A decline in insulin content was found to underlie the reduction in insulin secretion in old hom-E1506K islets. This reduction in islets insulin content is probably due to a

reduction in insulin production and not due to apoptosis of β -cells as the number of glucose-responsive β -cells observed by confocal Ca^{2+} imaging was unchanged between all genotypes.

Measurements of $[\text{Ca}^{2+}]_i$ in young het-E1506K islets showed spontaneous Ca^{2+} oscillations in 2 mM glucose in most islets, which did not result in an average elevation in $[\text{Ca}^{2+}]_i$. However, young hom-E1506K islets displayed vigorous Ca^{2+} oscillations in 2 mM glucose that led to an average elevation in $[\text{Ca}^{2+}]_i$. This suggests that in the hom-E1506K mice the mutant K_{ATP} channel is already closed in 2 mM glucose, causing membrane depolarisation and influx of Ca^{2+} . Confocal Ca^{2+} imaging revealed that there was no difference in the synchronicity of Ca^{2+} oscillations between wild-type and het-E1506K hom-E1506K islets. Both old het-E1506K and old hom-E1506K islets exhibited $[\text{Ca}^{2+}]_i$ responses with similar magnitude, but did no longer show Ca^{2+} oscillations. Stimulation of het-E1506K or hom-E1506K islets with 10 mM glucose, 0.2 mM tolbutamide plus 10 mM glucose or 70 mM KCl revealed a reduced increase in $[\text{Ca}^{2+}]_i$ compared to wild-type islets indicating either a reduction in Ca^{2+} influx or an enhanced clearance of Ca^{2+} . In order to further investigate this, voltage-dependent inward currents were recorded from β -cells in intact het-E1506K islets. It revealed that the magnitude of the sustained current measured at the end of the depolarisation pulse was similar in wild-type and het-E1506K β -cells. However the peak current in het-E1506K islets was larger than in wild-type cells, but – untypically for mouse β -cells – contained a Na^+ current resulting from a shift in its inactivation towards a more positive membrane potential.

6 GENERAL DISCUSSION

The pancreatic β -cell secretes insulin in response to an increase in the blood glucose concentration. Because insulin is the only glucose-lowering hormone, β -cell dysfunction leads to impaired glucose homeostasis, with excess and uncontrolled insulin secretion causing hyperinsulinemia and reduced insulin secretion producing diabetes. In this thesis I used mouse models and the insulin secreting cell line MIN6 to

- 1) investigate the role of reactive oxygen species (ROS) on glucose-stimulated $[\text{Ca}^{2+}]_i$ and insulin secretion in a mouse model of glucose intolerance due to lack of nicotinamide nucleotide transhydrogenase (Nnt), and in MIN6 cells in which glutathione peroxidase 1 (GPx1) was silenced.
- 2) characterise mouse models of neonatal diabetes (ND) produced by an activating mutation (V59M) in the Kir6.2 subunit of the K_{ATP} channel.
- 3) examine a mouse model of hyperinsulinism of infancy (HI), caused by an loss-of-function mutation (E1506K) in the SUR1 subunit of the K_{ATP} channel.

6.1 THE ROLE OF ROS ON INSULIN SECRETION

The effects of Nnt and GPx1, both part of the antioxidant defence mechanism of the β -cell, on glucose-stimulated changes in $[\text{Ca}^{2+}]_i$ and insulin secretion were investigated in Chapter 3.

Loss of Nnt function is linked to uncoupling of β -cell mitochondrial metabolism, which leads to less ATP production, enhanced K_{ATP} channel activity, the failure of glucose to raise $[Ca^{2+}]_i$ and eventually reduced insulin secretion (Freeman, Shimomura et al. 2006).

Nnt is a nuclear-encoded transmembrane protein, located in the inner mitochondrial membrane that utilises the mitochondrial membrane potential to generate NADPH from NADH (Hoek and Rydstrom 1988). NADPH is essential for the enzymatic regeneration of reduced glutathione, which in turn is used for the removal of ROS by several enzymes, including glutathione peroxidases (GPxs). To date, however, the exact nature of ROS and the mechanism of its detoxification are unknown.

I found that MIN6 cells in which Nnt was silenced displayed a greater increase in mitochondrial H_2O_2 upon stimulation with the ROS generator menadione or with 20 mM glucose than control cells. However, knockdown of GPx1 had no effect on mitochondrial H_2O_2 and was linked to a Ca^{2+} -independent hypersecretion of insulin. GPx1 is believed to play a pivotal role in removing H_2O_2 from the mitochondria. Although it is possible that ablation of GPx1 triggers an adaptive response resulting in upregulation of defence systems and resistance to higher levels of oxidative stress, the GPx1 knockdown could lead to an increase in cytosolic H_2O_2 which may function as a signalling molecule to stimulate insulin secretion downstream of Ca^{2+} influx.

The precise role of ROS, and in particular the role of H_2O_2 , in the process of insulin secretion is controversial, with some studies reporting a stimulatory effect (both dependent and independent of Ca^{2+}) and others an inhibitory effect (Maechler, Jornot et al. 1999; Pi, Bai et al. 2007; Gier, Krippeit-Drews et al. 2009; Leloup, Turrel-Cuzin et al. 2009; Rebelato, Abdulkader et al. 2010). How can these findings be reconciled? The impact ROS has on cellular physiology depends on several factors such as (1) the exact type of ROS, (2) the cell type and its unique defence mechanisms for coping with ROS challenges, (3) the amount and (4) the time of exposure. Even under similar experimental conditions, differences in sample preparation or

handling can impose oxidative stress on cells. For example, cell culture media are often deficient of antioxidants or contain free metal ions that could catalyse the formation of damaging hydroxyl radicals (Halliwell and M.C. Gutteridge 2007). All these points have to be considered when a comparison between different studies is made.

Exogenous GSH was previously reported to stimulate insulin secretion *in vivo* and *in vitro* (Ammon and Mark 1985; Del Guerra, Lupi et al. 2005). I found that exogenous GSH did not increase the glucose-stimulated rise in $[Ca^{2+}]_i$ in islets isolated from either wild-type mice or mice carrying Nnt mutations (N68K, G745D or C57BL/6J mice). This might suggested that GSH exerts its stimulating effect on glucose-stimulated insulin secretion downstream of Ca^{2+} influx. However, this hypothesis is confounded by the fact that the effect on Nnt on insulin secretion was lost in the mouse I studied.

Previous studies showed that ablation of Nnt in MIN6 cells (Freeman, Shimomura et al. 2006) or isolated β -cells (Toye, Lippiat et al. 2005) resulted in a complete loss of the glucose-stimulated increase in $[Ca^{2+}]_i$. By contrast, I found that the glucose-stimulated increase in $[Ca^{2+}]_i$ was not impaired in Nnt mutant islets but was similar to wild-type islets or to islets overexpressing Nnt (DBA/2 mouse strain). This discrepancy between my data and that of previous authors may be attributable to a change in the genetic background of the Nnt mutant mice. Unbeknownst to us, the Harwell animal house staff changed the strain of C3H mice used as wild-type controls for functional studies and for breeding pairs (without informing the scientists). This was because they desired a mouse with larger ears to facilitate ear-clipping for genotyping purposes. This only came to light after my experiments had been completed and I had unexpectedly found no effect of the Nnt mutations on glucose-dependent $[Ca^{2+}]_i$ responses. Other members of the laboratory then re-examined insulin secretion from isolated islets and discovered that there was no longer a difference between wild-type and mutant mice. Similarly, intraperitoneal glucose tolerance tests were not different: this appeared to be because the wild-

type mice now more closely resembled the mutant mice. It was not possible to repeat the experiments on the previous genetic background as these mice had all been sacrificed by the animal facility. The possibility of a background dependency of the Nnt mutation is supported by recent studies (Aston-Mourney, Wong et al. 2007; Wong, Blair et al. 2010).

6.2 MOUSE MODELS OF NEONATAL DIABETES EXPRESSING A GAIN-OF-FUNCTION MUTATION (V59M) IN THE KIR6.2 SUBUNIT OF THE K_{ATP} CHANNEL

In Chapter 4 mouse models of neonatal diabetes (ND) expressing a gain-of-function mutation (V59M) in the gene encoding the Kir6.2 subunit of the K_{ATP} channel was investigated. The Kir6.2-V59M mutation causes both a reduction in ATP sensitivity and an increased channel open probability (Proks, Antcliff et al. 2004), and gives rise to diabetes with muscle hypotonia and developmental delay in humans (Gloyn, Cummings et al. 2004). Transgenic mice constitutively expressing the V59M mutation in their β -cells (β -V59M mice) recapitulate the human disease: they exhibit a severe diabetic phenotype including the marked reduction of both first- and second-phase glucose-dependent insulin secretion from pancreatic islets (Girard, Wunderlich et al. 2009). I showed that this secretion profile was paralleled by a complete loss of the initial rise in $[Ca^{2+}]_i$ in β -V59M islets upon glucose stimulation. However, islets from ROSA mice (carrying a non-expressing V59M Kir6.2 gene) or from RIP-Cre mice (expressing Cre recombinase alone) showed no impairment in their $[Ca^{2+}]_i$ responses.

If expression of the Kir6.2-V59M transgene is induced in 12-week-old mice (ib-V59M), they rapidly become severely diabetic. Islets isolated from ib-V59M mice displayed a loss of the initial rise in glucose-stimulated $[Ca^{2+}]_i$ and a reduction in the sustained phase of the $[Ca^{2+}]_i$ response. This corresponded to a complete loss of insulin secretion (first- and second-phase) in ib-V59M islets.

In vivo administration of the K_{ATP} channel blocker glibenclamide was able to provide glycemic control in ib-V59M mice. In the presence of glibenclamide, both wild-type and ib-V59M islets showed elevated $[Ca^{2+}]_i$ at basal (2 mM) glucose. Subsequent application of 20 mM glucose produced a transient fall in $[Ca^{2+}]_i$ which then rose to levels similar to that found in 2mM glucose. The $[Ca^{2+}]_i$ responses of ib-V59M islets were paralleled by elevation of basal insulin secretion and restoration of glucose-stimulated first and second-phase secretion. Confocal imaging revealed that β -cells of ib-V59M islets responded to 20 mM glucose with non-synchronised Ca^{2+} oscillations across the islet. Conversely, in the presence of glibenclamide, Ca^{2+} oscillations in both 2 mM glucose and 20 mM glucose were synchronous in ib-V59M islets. Furthermore, the number of β -cell per islet responding to glucose with elevation of $[Ca^{2+}]_i$ was similar to control islets.

These findings on ib-V59M mice also demonstrated that some of the phenotypic changes observed in β -V59M mice, such as abnormal islet morphology and the reduction in β -cell number, are secondary to the expression of the Kir6.2-V59M mutation and are possibly mediated by long-term hyperglycemia. This illustrates the importance of early glibenclamide treatment to avoid the progressive loss of β -cell function and/or mass in human patients.

6.3 A MOUSE MODEL OF HYPERINSULINISM OF INFANCY EXPRESSING A LOSS-OF-FUNCTION MUTATION (E1506K) IN THE SUR1 SUBUNIT OF THE K_{ATP} CHANNEL

In Chapter 5 a mouse model of a dominant form of hyperinsulinism of infancy was investigated. This mouse model expresses a loss-of-function mutation (E1506K) in the gene encoding the SUR1 subunit of the K_{ATP} channel. The SUR1-E1506K mutation causes a reduction in channel activity that is mediated by the lack of MgADP activation (Huopio, Reimann et al. 2000). Patients heterozygous for this mutation show hypoglycemia early in life and tend to develop glucose

intolerance and diabetes later in life (Huopio, Reimann et al. 2000; Pinney, MacMullen et al. 2008; Vieira, Bergamin et al. 2009).

I found that islets isolated from young mice heterozygous for SUR1-E1506K (het-E1506K) displayed enhanced glucose-stimulated first- and second-phase insulin secretion, whereas young islets homozygous for SUR1-E1506K (hom-E1506K) exhibited enhanced basal secretion and elevated first- and second-phase insulin secretion. In het-E1506K islets isolated from old mice, glucose-stimulated insulin secretion had dropped to normal levels. In old hom-E1506K islets, basal insulin secretion was normal while glucose-stimulated insulin secretion was markedly reduced in comparison to wild-type islets. This reduction in insulin secretion was due to a decline in the insulin content of old hom-E1506K islets. Confocal Ca^{2+} imaging revealed that the number of glucose-responsive β -cells was similar between hom-E1506K and wild-type islets, suggesting that the reduction in islet insulin content is not due to loss of β -cells but probably caused by a reduction in insulin production.

Measurements of $[\text{Ca}^{2+}]_i$ in young het-E1506K islets showed spontaneous Ca^{2+} oscillations in 2 mM glucose in most islets, which did not result in an elevation of the averaged $[\text{Ca}^{2+}]_i$. However, young hom-E1506K islets displayed vigorous Ca^{2+} oscillations in 2 mM glucose that led to an increase in the time-averaged $[\text{Ca}^{2+}]_i$. This suggests that in hom-E1506K mice the mutant K_{ATP} channel is already closed in 2 mM glucose, causing membrane depolarisation and influx of Ca^{2+} . Both old het-E1506K and old hom-E1506K islets exhibited $[\text{Ca}^{2+}]_i$ responses of similar magnitude, but no longer showed Ca^{2+} oscillations. Confocal Ca^{2+} imaging revealed that 10 mM glucose stimulation produced synchronous Ca^{2+} oscillations between single β -cells in het-E1506K or hom-E1506K islets, indicating no impairment in pulsatile insulin secretion.

Stimulation of het-E1506K or hom-E1506K islets with 10 mM glucose produced a smaller increase in $[\text{Ca}^{2+}]_i$ than in wild-type islets. This corresponded to an increase in first- and second-phase insulin secretion from these islets, indicating an enhanced amplifying pathway.

Furthermore, depolarisation of young het-E1506K islets with tolbutamide or KCl revealed the $[Ca^{2+}]_i$ transient was smaller than in wild-type islets. This suggests that in het-E1506K islets either Ca^{2+} influx is reduced or the clearance of Ca^{2+} is enhanced. In order to investigate this further, voltage-dependent inward currents were recorded from β -cells in intact het-E1506K islets. The results revealed that the magnitude of the sustained current measured at the end of the depolarisation pulse was similar in wild-type and het-E1506K β -cells. However the peak inward current in het-E1506K islets was larger than in wild-type cells, but – atypically for mouse β -cells – contained a large Na^+ component that resulted from a shift in the voltage-dependence of inactivation towards more positive membrane potentials.

6.4 CLINICAL RELEVANCE AND IMPLICATIONS OF THE DIFFERENT MOUSE MODELS

Their genetic and physiological similarity to humans has made the mouse a valuable model for biomedical research on diabetes and hyperinsulinemia. In addition to genetic technologies that enable manipulation of the mouse's genome, environmental factors such as physical activity or food intake can easily be controlled in the laboratory.

6.4.1 THE NNT MOUSE MODEL

A human counterpart for the mouse model expressing a non-functional Nnt is still missing. So far, no mutations in the Nnt gene or other mitochondrial enzymes have been reported in human type 2 diabetic patients (Aston-Mourney, Wong et al. 2007; Remedi and Nichols 2009). However, my studies suggest that in Nnt-mutant mice physiological processes such as glucose-stimulated $[Ca^{2+}]_i$ responses, insulin secretion and glucose intolerance depend on the genetic background. This idea is corroborated by recent research on Nnt mutant mice (Aston-Mourney, Wong et al. 2007;

Wong, Blair et al. 2010). The importance of genetic background on the phenotype indicates that the function of the *Nnt* gene may be modified by other genes (Nadeau 2001).

That modifying genes can play an important role in phenotype expression is exemplified by cystic fibrosis, one of the most common human autosomal recessive diseases. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*), which encodes a chloride ion channel (Nadeau 2001). The clinical features of cystic fibrosis, such as mucus accumulation in the airways, impaired salt resorption in sweat glands, chronic bronchopulmonary infections and infertility, are variable in their the age of onset and their severity (Zielenski and Tsui 1995). Moreover, the lifespan of *CFTR*-deficient mice depends on the genetic background, which indicates that modifying genes modulate the severity of the disease. Indeed, Rozmahel et al. have identified a cystic fibrosis modifying gene, which possibly encodes a calcium-activated chloride conductance channel, that restores relatively normal membrane electrophysiology (Rozmahel, Wilschanski et al. 1996). Their study illustrates the importance of modifying genes in mouse models of human disease: it was one of the first examples of the identification of a modifying gene in mice that was then also found in humans (Nadeau 2001).

6.4.2 THE NEONATAL DIABETES MOUSE MODELS

Neonatal diabetes is most commonly caused by heterozygous gain-of-function mutations in the *ABCC8* and *KCNJ11* genes which encode the SUR1 and Kir6.2 subunits of the pancreatic β -cell K_{ATP} channel, respectively (Gloyn, Cummings et al. 2004; Babenko, Polak et al. 2006). Gain-of-function mutations open the K_{ATP} channel independently of glucose metabolism, resulting in a decrease in the electrical excitability of the β -cell. This leads to a reduced $[Ca^{2+}]_i$ transient and reduced insulin secretion in response to a glucose challenge. Mice expressing the Kir6.2-V59M gain-of-function mutation in their β -cells recapitulate many of the features of neonatal diabetes in humans. By 5

weeks of age the blood glucose levels of these mice were substantially increased and plasma insulin was undetectable (Girard, Wunderlich et al. 2009). Furthermore, isolated V59M islets exhibited reduced insulin secretion and showed a marked reduction of insulin content, accompanied by a loss of β -cell mass. This mouse model clearly shows how reduced metabolism-excitation coupling leads to decreased $[Ca^{2+}]_i$ and insulin secretion and how neonatal diabetes can result simply from a loss of insulin secretion.

Sulphonylureas, such as tolbutamide or glibenclamide, bind to the SUR1 subunit of the K_{ATP} channel, leading to reduced channel activity, membrane depolarization, and insulin secretion independently of the metabolic state of the β -cell (Gribble and Reimann 2002). Most neonatal diabetes patients are now treated with sulphonylureas (Ashcroft 2010). Sulphonylurea therapy has not only the advantage of improving the patients' quality of life and managing their clinical symptoms: it also makes their β -cells susceptible to various factors that potentiate insulin secretion once $[Ca^{2+}]_i$ is elevated. As a consequence, the amplifying pathway of glucose-stimulated insulin secretion (which is explained in detail in Chapter 1) is enabled. Other pathways that augment insulin secretion are mediated by incretin hormones such as glucagon-like peptide-1 (GLP-1) or neurotransmitters, such as acetylcholine. These should also be enhanced following drug therapy.

GLP-1 is secreted from intestinal K cells in response to food in the gut (Baggio and Drucker 2007). Binding of GLP-1 to its specific receptor on pancreatic β -cells leads to activation of adenylate cyclase with results in the production of intracellular cAMP and stimulation of exocytosis (Gromada, Ding et al. 1997). Secretion of acetylcholine from pancreatic nerves, triggered by the intake or anticipation of food, activates muscarinic M3 receptors on the β -cell and stimulates several effector proteins including phospholipase C (PLC) and protein kinase C (PKC) (Gilon and Henquin 2001). Operation of these pathways in ND patients treated with sulphonylureas might explain why oral glucose is far more effective than intravenous glucose at

triggering insulin secretion (Pearson, Flechtner et al. 2006). The importance of these potentiating pathways is that they couple the secretion of insulin to food intake in neonatal diabetes patients treated with sulphonylureas.

6.4.3 THE HYPERINSULINEMIA OF INFANCY MOUSE MODEL

The most common cause of hyperinsulinemia of infancy (HI) is loss-of-function mutations in the β -cell K_{ATP} channel subunits (Thomas, Cote et al. 1995; Thomas, Ye et al. 1996). In HI, reduced or absent K_{ATP} channel activity is expected to result in constitutive membrane depolarization independent of glucose metabolism. This leads to elevated $[Ca^{2+}]_i$ and hypersecretion of insulin (Ashcroft 2005; Flanagan, Clauin et al. 2009). Huopio et al. first reported that patients heterozygous for the SUR1-E1506K mutation show hypoglycemia early in life and tend to develop glucose intolerance and diabetes later in life.

Perhaps surprisingly, het-E1506K islets isolated from young mice did not display elevated basal insulin secretion. However, glucose-stimulated insulin secretion in these islets was elevated, which may account for the HI phenotype of patients heterozygous for the mutation. In contrast to het-E1506K islets, young hom-E1506K islets exhibited elevated basal insulin secretion as well as enhanced glucose-stimulated secretion. The transition from hyperinsulinemia to glucose intolerance and diabetes seen in patients, seems to be recapitulated by the SUR1-E1506K mouse model, as glucose-stimulated insulin secretion measured from older het-E1506K islets declined to normal levels, and the reduction in insulin secretion was even more pronounced from old hom-E1506K islets. Collectively, this indicates that the clinical phenotype of the human heterozygous SUR1-E1506K mutation seems to be better represented by the homozygous mouse model.

Mice lacking K_{ATP} channels because of knockout of either of the genes that encode the K_{ATP} channel subunits (SUR1 and Kir6.2) have also been generated to model human HI (Miki,

Nagashima et al. 1998; Seghers, Nakazaki et al. 2000). These mice, however, do not completely replicate the HI phenotype in humans: although hypersecretion of insulin occurs immediately after birth, mice develop glucose intolerance within a few days. This is linked to a reduced insulin secretion from isolated islets, as reported by most laboratories (Miki, Nagashima et al. 1998; Seghers, Nakazaki et al. 2000; Shiota, Larsson et al. 2002; Dufer, Haspel et al. 2004; Li, Buettger et al. 2004; Nenquin, Szollosi et al. 2004; Sieg, Su et al. 2004; Szollosi, Nenquin et al. 2007; Ravier, Nenquin et al. 2009). Despite the lower insulin secretion Kir6.2- or SUR1-knockout β -cells are depolarized at low glucose concentrations and show elevated $[Ca^{2+}]_i$. This would suggest that the impairment of secretion occurs at a stage downstream of Ca^{2+} influx. The mechanism for this reduced insulin secretion is unknown. Although some studies reported a decrease in insulin content from SUR1 knockout mice (Nakazaki, Crane et al. 2002; Shiota, Larsson et al. 2002; Nenquin, Szollosi et al. 2004), others found that the smaller secretory responses were not attributable to differences in islet insulin content (Eliasson, Ma et al. 2003; Haspel, Krippeit-Drews et al. 2005; Remedi, Rocheleau et al. 2006; Szollosi, Nenquin et al. 2007; Gier, Krippeit-Drews et al. 2009; Marhfour, Moulin et al. 2009; Ravier, Nenquin et al. 2009). This may be related to differences in culture conditions, or the use of fresh versus cultured islets. The group of Jean-Claude Henquin reported that basal insulin secretion and secretory responses to glucose from K_{ATP} knockout islets exceeded those of wild-type islets when islets were incubated overnight in 10 mM or 15 mM glucose, whereas this was not seen following overnight culture in 5 mM glucose or when using freshly isolated islets (Szollosi, Nenquin et al. 2007; Ravier, Nenquin et al. 2009).

Upon stimulation with GPL-1, intracellular cAMP levels were similar between SUR1 knockout and wild-type islets, but the PKA-independent component of insulin secretion (which involves cAMP-activated guanine nucleotide exchange factors for Ras-like GTPases (Epac2)) was absent in the SUR1 knockout islets (Nakazaki, Crane et al. 2002; Eliasson, Ma et al. 2003). This raises the possibility that SUR1, which is known to bind Epac2 (Ozaki, Shibasaki et al. 2000), is involved in the exocytosis of insulin. It was therefore proposed that in normal β -cells the SUR1

subunit of the K_{ATP} channel recruits Epac2 and downstream effector proteins including RIM2, Piccolo or Rab proteins to the plasma membrane, where a net of protein–protein interactions may play an important part in the exocytosis of insulin secretory granules (Eliasson, Ma et al. 2003; Holz, Kang et al. 2006).

Several studies have shown that blocking the K_{ATP} channel in wild-type islets with glibenclamide produced similar physiological responses to those observed in K_{ATP} channel knockout islets. Likewise, this thesis demonstrates that the glucose-stimulated $[Ca^{2+}]_i$ responses in islets incubated with glibenclamide are identical to K_{ATP} knockout islets. Dufer et al. showed that reduced K_{ATP} channel activity caused by SUR1 knockout or sulphonylurea block similarly affected Ca^{2+} uptake into non-ER intracellular organelles (Dufer, Haspel et al. 2007). Upregulation of antioxidant enzymes and reduced sensitivity of SUR1 knockout β -cells to H_2O_2 -induced apoptosis were paralleled by wild-type β -cells treated with the sulphonylureas (Gier, Krippeit-Drews et al. 2009). Furthermore, the responsiveness to amino acid (Li, Nissim et al. 2008) or acetylcholine (Doliba, Qin et al. 2004) stimulation was equally enhanced in SUR1 knockout and glibenclamide-treated wild-type islets. When wild-type islets (Fuhlendorff, Rorsman et al. 1998) or islets isolated from *ib-V59M* mice were incubated with glibenclamide their basal and glucose-stimulated insulin secretion increased. This contrasts with K_{ATP} channel knockout islets, in which insulin secretion was reduced, probably due to the role of the K_{ATP} channel in the insulin secretory machinery, as discussed above.

Patients with type 2 diabetes chronically treated with sulphonylureas often require insulin treatment because of a failure of their β -cells to secrete insulin (Matthews, Cull et al. 1998). This situation was replicated in mice treated with glibenclamide (Remedi and Nichols 2008). In this study it was shown that wild-type mice in which a glibenclamide pellet was implanted under their skin initially displayed lower blood glucose levels and improved glucose tolerance than control animals. However, after 1 week of treatment, these mice were as glucose

intolerant as adult K_{ATP} knockout mice, and insulin secretion from isolated islets was markedly reduced. Interestingly, the decreased capacity for insulin secretion was fully restored in these islets within hours of drug washout *in vitro*, or within 1 month after drug withdrawal *in vivo*. The underlying mechanisms for the secretory failure and its rapid onset are unknown. The study, however, indicates that failure to secrete insulin in response to long term glibenclamide treatment is not due to β -cell apoptosis or reduced β -cell mass, and that it is quickly reversible. These findings suggest that it might be possible to prevent or reverse secretory failure caused by chronic sulphonylurea treatment in type 2 diabetic patients by using short-acting sulphonylureas.

In conclusion, this thesis shows that mouse models can help to understand the pathophysiology of diabetes and hyperinsulinemia and may aid the development of new treatments. However, it also reveals limitations of some mouse models and illustrates that they cannot replace research on human tissue or patients.

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