‘DYNAMICS OF INSULIN SECRETION’

This thesis is submitted for the degree of Doctor in Medicine (MD) to

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by

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DECLARATION

I declare that I composed this thesis and that I performed all the experiments described in this thesis. Furthermore, I analysed and interpreted the results that arose from these experiments. This thesis has not been submitted for any other degree, diploma or professional qualification.

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Dedication

This work is dedicated to my beloved parents, Lee Eng and Song Lam Hwa who gave their constant and tireless support as without their encouragement, this work will not be possible.
Publications


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Abstracts

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2. Song SH, McIntyre SS, Rhodes CJ, Veldhuis JD, Butler PC. Diazoxide attenuates glucose-induced defects in first phase insulin release and pulsatile insulin secretion in human islets Diabetes 2000; (Suppl 1): A419. (This abstract was presented at the 60th American Diabetes Association meeting in San Antonio, Texas.)
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THESIS ABSTRACT

Type 2 diabetes mellitus is characterised by relative insulin deficiency. Furthermore, the pattern of insulin secretion is abnormal, with reduced first and second phase insulin secretion, increased irregularity of insulin release and diminished insulin pulses. Previously it has been reported that β-cell rest (in the form of transient inhibition of insulin secretion) improves insulin secretion in humans with Type 2 diabetes mellitus and animal models of diabetes. The specific mechanism through which this was achieved remains unknown. In this MD thesis, I undertook four related projects that address the pattern of insulin secretion and the specific mechanism by which β-cell positively influences these.

Project one: Direct measurement of the pattern of insulin release in the portal vein (immediately downstream of the pancreas) in human subjects in the basal state and in response to glucose stimulation.

Project two: Examination of the effects of acute inhibition of insulin secretion in humans with Type 2 diabetes by diazoxide on the pattern of insulin secretion.

Project three: Development and validation of a method to quantify pulsatile insulin release by cultured human islets in an open loop perifusion system.

Project four: Use of the system developed and validated in project 3 to test the hypothesis that transient β-cell rest in human islets induced by diazoxide (inhibiting insulin secretion by opening β cell potassium channels) prevents loss of first phase insulin release and pulsatile insulin secretion caused by culture of the islets in glucose concentrations comparable to those seen in Type 2 diabetes.
In these experiments, the following observations were made; (1) when insulin secretion was measured directly in the portal circulation in human subjects, hyperglycaemia enhanced insulin secretion through the specific mechanism of amplification of the secretory burst mass while the insulin pulse frequency remained unchanged; (2) acute partial inhibition of insulin secretion by diazoxide in patients with Type 2 diabetes did not effect the regularity of insulin secretion but did decrease the insulin clearance rate; (3) a deconvolution programme was established that was able to detect 90% of insulin pulses delivered by single human islets and (4) using the novel islet perfusion system it was possible to show that human islets cultured at high glucose concentrations lost first phase insulin secretion and insulin pulse amplitude but these were restored in islets exposed to a transient period of β-cell rest.

The overall conclusion of these studies is that the dynamics of insulin secretion are important as well as the absolute secretion rate and that the abnormalities in these dynamics may be related in part to β cell insulin stores that can be manipulated by transient β cell rest.
Chapter 1

Pulsatile insulin secretion – a literature review
Introduction

The neuro-endocrine system secretes hormones in pulsatile manner (1,2). Pulsatile hormone secretion has been shown to improve target organ sensitivity (3,4). Disrupted function of the hypothalamo-pituitary axis is often characterised by perturbation of the pulsatile pattern of secretion (5). The endocrine pancreas has many properties in common with the hypothalamo-pituitary axis including pulsatile release of hormones. Oscillations of the plasma insulin concentration were first shown by Goodner et al who reported insulin oscillations with periodicity of 10 minutes in rhesus monkeys (6). A similar oscillatory pattern has been subsequently demonstrated in vivo in humans (7), dogs (8) and baboons (9) and in vitro, in the isolated canine pancreas (10), groups of perifused human islets (11), single rat (12) and mouse (13) islets and single human β-cells (14). In the fasting state, the majority of insulin (at least 70%) is released in the pulsatile mode (8,15) and the regulation of insulin secretion occurs via modulation of this pulsatile component (16-19). Moreover, abnormal patterns of pulsatile insulin secretion have been reported in patients with type 2 diabetes mellitus (20) and their first-degree relatives with normal (21) and abnormal (22) glucose tolerance implying that the disruption of the oscillatory pattern of insulin secretion may play an important role in the pathogenesis of this condition.

Physiology of insulin secretion

The β-cell secretes insulin in response to both neurohormonal (such as acetylcholine) and nutrient stimuli (23,24). The signal transduction of these two classes of agonist is distinct since the former interacts with a plasma membrane
receptor whereas the latter is initiated by intracellular metabolism of the nutrients (23,24). Various nutrients have the ability to stimulate insulin release. This includes glucose, amino acids, free fatty acids (FFA) and ketone bodies (23). Of these, glucose is the major physiological stimuli.

The pathway of glucose-induced insulin secretion has been extensively studied. Since the identification of the ATP-sensitive potassium (K_{ATP}) channel in 1984 (25), this channel was found to be involved in the mechanism of glucose-induced insulin secretion (26-28). This pathway is referred to as the K_{ATP} channel-dependent pathway. The mechanism by which glucose stimulates insulin secretion in this pathway is as follows (29-31) (figure 1); glucose is transported into the β-cell via glucose transporter, enters the glycolytic pathway and phosphroylated to glucose-6-phosphate by glucokinase. This step consumes ATP and generates ADP resulting in transient decrease in ATP/ADP ratio. This has the dual effect of stimulating glycolysis via activation of phosphofructokinase (PFK) and mitochondrial metabolism via activation of mitochondrial dehydrogenases and the respiratory chain. This results in increased rates of glycolysis and oxidative phosphorylation leading to the elevation of ATP/ADP ratio which in turn, closes the K_{ATP} channel causing membrane depolarisation (32), influx of extracellular Ca^{2+} via L-type voltage-gated Ca^{2+} channels (33) and a rise in cytosolic Ca^{2+} concentration [Ca^{2+}]_{i} (34). The elevation of [Ca^{2+}]_{i} activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) which phosphorylates proteins involved in the exocytosis of insulin granules resulting in insulin secretion (35).
Figure 1

Model of the regulation of insulin secretion from β-cell by cytosolic ATP/ADP ratio and K⁺-ATP channel closure. Abbreviations: GK – glucokinase; G6P – glucose-6-phosphate; F6P – fructose-6-phosphate; PFK – phosphofructokinase; PK – pyruvate kinase; CaMK11 – Ca²⁺/calmodulin-dependent protein kinase 11; Ψ – membrane potential; [Ca²⁺], – cytoplasmic Ca²⁺ concentration.
However, it became apparent that the $K_{ATP}$ channel-dependent pathway only partially accounted for the mechanism of insulin secretion (36). Stimulation with glucose produced far greater insulin response especially during the second phase secretion than high concentration of KCl or sulphonylurea, both of which caused simple membrane depolarisation. Furthermore, an increase in $[Ca^{2+}]_i$ did not reproduce the biphasic response induced by glucose. These observations implied the existence of additional mechanisms by which glucose could enhance insulin secretion. In 1992, it was demonstrated that glucose strongly augmented insulin secretion when the $K_{ATP}$ channel was kept in the open state by diazoxide and the plasma membrane depolarised by high KCl concentration to increase $[Ca^{2+}]_i$. This demonstrated that glucose could stimulate insulin release independently of the $K_{ATP}$ channel in presence of elevated $[Ca^{2+}]_i$. This pathway was referred to as $K_{ATP}$ channel-independent, $Ca^{2+}$- dependent pathway (37-39). Moreover, glucose was also found to augment insulin secretion in $Ca^{2+}$-depleted islets in the complete absence of extracellular $Ca^{2+}$ and in absence of any rise in $[Ca^{2+}]_i$ (40). This mechanism of glucose augmentation functioned maximally when protein kinase A (PKA) and protein kinase C (PKC) in the β-cell were activated simultaneously. This pathway was referred to as $K_{ATP}$ channel-independent, $Ca^{2+}$- independent pathway. Substances such as GLP-1 (which activates PKA) (41) and acetylcholine and cholecystokinin (which activate PKC) (24,33,42) augment glucose-induced insulin secretion through this mechanism. It required the binding of these agonists with their receptors on the β cell membrane and activation of G-protein. Apart from their differential requirement for $Ca^{2+}$, these two $K_{ATP}$ channel-independent pathways have common characteristics (36); (a) dependence on glucose metabolism, (b) do
not initiate but only augment insulin release, (c) similar magnitude of glucose-induced insulin release, (d) similar glucose concentration-response curves, (e) similar temporal profiles that resemble the second phase of glucose-induced insulin release, (f) stimulated by metabolisable nutrients other than glucose and (g) stimulated by free fatty acids.

At what stage in the stimulus-secretion pathway do the $K_{ATP}$ channel-independent pathways become involved in the augmentation of insulin secretion? It has been proposed that the site of entry is located at the final stages of insulin secretion, namely, the exocytosis of immediately releasable pool of insulin (36). Thirty years ago, Grodsky proposed a two-compartmental insulin storage system within the $\beta$ cell (48). Since then, it has been confirmed that insulin is indeed stored in two pools; reserve and immediately releasable insulin pool. This was demonstrated by capacitance measurement of exocytosis (49) and identification of proteins involved in the docked pool of immediately releasable insulin granule (50). Exocytosis of immediately releasable pool of insulin is regulated by $Ca^{2+}$ and GTP independently (49). $Ca^{2+}$-dependent augmentation pathway stimulates $Ca^{2+}$-triggered exocytosis while $Ca^{2+}$-independent augmentation pathway stimulates GTP-triggered exocytosis (36).

It has been proposed that the $K_{ATP}$ channel-independent ($Ca^{2+}$-dependent and independent) pathways act through a common mechanism to augment glucose-induced insulin secretion, namely, an increase in the level of cytosolic long chain acyl-CoA (LC-CoA) esters (43,44) (figure 2). Following stimulation with glucose,
Figure 2
Mechanism by which $K^+_{ATP}$ channel-independent pathway augments insulin secretion. Glucose metabolism leads to elevation of cytosolic malonyl CoA levels, inhibition of mitochondrial CPTI and consequent inhibition of fatty acid oxidation. This results in an increase in long chain acyl-CoA esters. Abbreviation: CPTI – carnitine palmitoyl-transferase I; Fa-CoA – fatty acyl CoA; FFA – free fatty acid
there is an increase in cytosolic malonyl-CoA which inhibits mitochondrial enzyme carnitine palmitoyltransferase I leading to decreased fatty acid oxidation and increased cytosolic LC-CoA levels (45). Cytosolic LC-CoA esters then augment insulin secretion by modulating the activity of enzymes and other transducing effectors involved in insulin secretion. For example, this can occur through activation of PKC directly or indirectly via diacylglycerol production (46,47) or acylation of key proteins in the exocytosis of insulin granule (45).

Finally, both $K_{ATP}$ channel-dependent and $K_{ATP}$ channel-independent ($Ca^{2+}$-dependent and independent) pathways are needed to cause the full effect of glucose-induced insulin secretion (figure 3). In the in vivo condition, the pancreatic $\beta$-cells are exposed to various secretagogues, hormones, peptides and neurohormonal agents apart from glucose. These agents exert their influence on insulin secretion via $K_{ATP}$ channel-independent ($Ca^{2+}$-dependent and independent) pathways and function synergistically with $K_{ATP}$ channel-dependent pathway to exert the full magnitude of insulin secretion.

**Location and origin of pacemaker activity for oscillatory insulin secretion**

After Goodner et al demonstrated oscillatory plasma insulin concentrations, several possibilities to account for this were considered (6). Oscillations in plasma insulin concentration could be due to intermittent clearance or secretion of insulin. However, since the plasma C-peptide concentration also oscillates in phase with insulin (51), the origin of insulin oscillations was ascribed to pulsatile secretion rather than clearance. The other possibility that may lead to the pulsatile release of
Glucose-induced insulin release from β-cell. Full magnitude of insulin secretion requires augmentation of insulin release from the $K_{ATP}$ channel independent pathway.
insulin is intermittent input to islets from the central nervous system and/or oscillations in the plasma glucose concentration or other circulating secretagogues. However, as pulsatile insulin release was observed in isolated canine pancreas perfused with buffer at a constant glucose concentration, clearly, pulsatile insulin secretion does not require either input from the central nervous system or oscillations of circulating glucose concentration (10). This insight was reinforced by the observation that isolated islets of Langerhans exposed to constant glucose concentration secrete insulin in a pulsatile manner (52). These data suggest that the pacemaker for pulsatile insulin secretion lies within the pancreas and that it may be one or more islets of Langerhans. Currently, there are several hypotheses to account for the pacemaker activity within the pancreas.

(1) **Ganglia hypothesis**

It has been proposed that intra-pancreatic ganglia and associated nerves may act as a pacemaker and/or as a co-ordination system to generate oscillatory insulin release (53). This hypothesis is supported by several observations. Firstly, pancreatic ganglia and the associated nerves do not degenerate after denervation of the pancreas suggesting that they possess some form of autonomous function (54). Secondly, there is a close anatomical relationship between the intrapancreatic ganglia, associated nerves and islets (55). Histological examination demonstrated that nerves originating from the ganglia connect both with other ganglia and groups of islets (56). β-cell secretion can be modulated by stimuli from nerves derived from the intrinsic ganglia or from secretion of non-β cells locally in the islet which may first receive nervous stimulation (57). Thirdly, intra-pancreatic ganglia have been
shown to display intrinsic electrical rhythmic activity consistent with frequency of pulsatile insulin secretion (53). Pancreatic ganglia receive innervation from adrenergic, cholinergic and peptidergic nerves (54). However, as blockade of adrenergic and muscarinic receptors did not affect insulin secretory pattern both in canine pancreas in vitro (10) and humans in vivo (58), peptidergic nerves might be the principal mediator of insulin secretion. It has been suggested that post-synaptic nicotinic receptors at the ganglia might be the principal regulator and co-ordinator of insulin secretion (59).

However, there are also arguments against a central role of intrapancreatic ganglia as the pacemaker for pulsatile insulin secretion. Pulsatile insulin secretion occurs in perifused single islets (12,13) and in single β-cells (14) revealing that β-cell has the properties to be an independent pacemaker. Currently, there are two hypotheses to account for the origin of oscillations in β-cells; oscillations in cytosolic Ca$^{2+}$ concentration, [Ca$^{2+}]_i$ and oscillations in glycolysis.

(2) Cytosolic [Ca$^{2+}]_i$ oscillation hypothesis

This hypothesis proposes that oscillations in cytosolic [Ca$^{2+}]_i$ drive the oscillations in insulin secretion. Following glucose stimulation, oscillations in membrane potential lead to intermittent Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels (60,61). This periodic influx of Ca$^{2+}$ leads to oscillations in cytosolic [Ca$^{2+}]_i$ and insulin secretion (62,63). The regulatory role of cytosolic [Ca$^{2+}]_i$ in insulin secretion is well-established. It has been shown that Ca$^{2+}$ omission (64,65) or addition of voltage-dependent Ca$^{2+}$ channel blocker (66) abolishes glucose-stimulated insulin
secretion. Furthermore, apart from glucose-induced elevation in cytoplasmic $[Ca^{2+}]_c$ with subsequent release of insulin from β-cells (62,63), elevation in the external Ca$^{2+}$ concentration has been shown to stimulate insulin secretion from permeabilized β-cells (67). The regulatory role of cytosolic $[Ca^{2+}]_c$ was further supported by the demonstration of the synchronous oscillations of cytosolic $[Ca^{2+}]_c$ and insulin secretion from pancreatic islets (68). More recently, the issue of synchronicity between cytosolic $[Ca^{2+}]_c$ and insulin secretion oscillations was further strengthened when Jonas et al (63) demonstrated a close temporal and quantitative relationship between $[Ca^{2+}]_c$ and insulin secretion in mouse pancreatic β-cells. Synchronous oscillatory insulin secretion occur only when $[Ca^{2+}]_c$ oscillates. When oscillations in $[Ca^{2+}]_c$ were abolished by continuous depolarisation with 30 mmol/L K$^+$ resulting in sustained elevation in $[Ca^{2+}]_c$, no oscillations in insulin secretion was seen. Further support for the regulatory role of cytosolic $[Ca^{2+}]_c$ came from the observation that imposed oscillations of free Ca$^{2+}$ in permeabilised insulin-secreting cells triggered oscillations in insulin secretion in absence of any metabolic oscillation (69).

However, in order to generate oscillations, a feedback system must exist to maintain cycles of depolarisation followed by repolarisation of membrane potential. Two mechanisms have been proposed;

(a) **Feedback of $[Ca^{2+}]_c$ on mitochondrial respiration** (figure 4)

This model depends on feedback loop between $[Ca^{2+}]_c$, mitochondrial respiration, its ATP production and the subsequent effect on ATP/ADP ratio and $K^{+}_{ATP}$ channel activity (70). After glucose stimulation, the rise in $[Ca^{2+}]_c$ is followed by Ca$^{2+}$ uptake
Glucose metabolism increases the ATP/ADP ratio. This leads to closure of $K_{\text{ATP}}$ channel (1) with depolarisation of the plasma membrane (2) and opening of the voltage-dependent $Ca^{2+}$ channel. Influx of extracellular $Ca^{2+}$ (3) causes a rise in cytoplasmic $[Ca^{2+}]_i$, which triggers exocytosis of insulin granules (4). With elevation of cytoplasmic $[Ca^{2+}]_i$, there is increased uptake of $Ca^{2+}$ into the mitochondria (5) which leads to decrease in the mitochondrial membrane potential and reduction in ATP production and ATP/ADP ratio (6). This leads to reopening of the $K_{\text{ATP}}$ channel, membrane repolarisation, arrest of $Ca^{2+}$ influx, lowering of $[Ca^{2+}]_i$; and restoration of a high ATP/ADP ratio to initiate a new cycle. Abbreviation: $\Psi$ - membrane potential of $\beta$-cell; $\Psi_m$ - membrane potential of mitochondria.
by mitochondria resulting in decrease in mitochondrial membrane potential and inhibition of ATP synthesis. This leads to a reduction in the ATP/ADP ratio causing the $K^+_{ATP}$ channels to open leading to membrane repolarisation and inactivation of the voltage-gated $Ca^{2+}$ channels. This causes $[Ca^{2+}]_i$ to decrease, thus, relieving its inhibition on mitochondrial respiration and ATP production. ATP/ADP ratio rises again to generate another cycle of membrane potential and $[Ca^{2+}]_i$ changes. This could trigger oscillations of the ATP/ADP ratio and its subsequent effect on $K^+_{-ATP}$ channel activity, membrane potential, $[Ca^{2+}]_i$ and insulin secretion through feedback inhibition by $Ca^{2+}$ of mitochondrial ATP production. However, experimental data do not support this hypothesis (71).

(b) Feedback of $[Ca^{2+}]_i$ on $K^+_{-ATP}$ channel activity (figure 5)

Alternatively, repolarisation may be achieved by feedback of $[Ca^{2+}]_i$ on $K^+_{ATP}$ channel activity via modulation of ATP/ADP ratio. Once $[Ca^{2+}]_i$ is elevated, further rise is prevented by the inhibitory actions of $Ca^{2+}$ on the activity of $K^+_{ATP}$ channels (72). A recent study showed that this might be achieved via changes in ATP consumption (73). The rise in $[Ca^{2+}]_i$ results in lesser degree of rise in the ATP/ADP ratio compared to situation where $[Ca^{2+}]_i$ is prevented from rising through blockade of extracellular $Ca^{2+}$ entry (73). Elevation in $[Ca^{2+}]_i$ causes simultaneous increment in ATP production through stimulation of $Ca^{2+}$-dependent mitochondrial dehydrogenases (74) and increment in ATP consumption possibly through membrane ATPase (75). However, there is net reduction ATP concentration as the ATP consumption occurs at a faster rate than its production (73). This leads to reduction in ATP/ADP ratio resulting in reopening of $K^+_{ATP}$ channels, membrane
Glucose metabolism increases the ATP/ADP ratio. This leads to closure of $K_{\text{ATP}}$ channel (1) with depolarisation of the plasma membrane (2) and opening of the voltage-dependent $\text{Ca}^{2+}$ channel. Influx of extracellular $\text{Ca}^{2+}$ (3) causes a rise in cytoplasmic $[\text{Ca}^{2+}]_i$ which, in addition to triggering exocytosis of insulin granules (4), exerts an effect that influence the ATP/ADP ratio in opposite direction. Via activation of mitochondrial dehydrogenases, $\text{Ca}^{2+}$ promotes ATP production (5) and also simultaneously increases ATP consumption (6). The net result is reduction in ATP/ADP ratio, leading to reopening of the $K_{\text{ATP}}$ channel, membrane repolarisation, arrest of $\text{Ca}^{2+}$ influx, lowering of $[\text{Ca}^{2+}]_i$, and restoration of a high ATP/ADP ratio to initiate a new cycle.

Abbreviation: $\Psi$ - membrane potential of β-cell.

**Figure 5**

Model of feedback control of $\text{Ca}^{2+}$ on the $K_{\text{ATP}}$ channel activity via modulation of ATP consumption and ATP/ADP ratio in β-cells. Glucose metabolism increases the ATP/ADP ratio. This leads to closure of $K_{\text{ATP}}$ channel (1) with depolarisation of the plasma membrane (2) and opening of the voltage-dependent $\text{Ca}^{2+}$ channel. Influx of extracellular $\text{Ca}^{2+}$ (3) causes a rise in cytoplasmic $[\text{Ca}^{2+}]_i$ which, in addition to triggering exocytosis of insulin granules (4), exerts an effect that influence the ATP/ADP ratio in opposite direction. Via activation of mitochondrial dehydrogenases, $\text{Ca}^{2+}$ promotes ATP production (5) and also simultaneously increases ATP consumption (6). The net result is reduction in ATP/ADP ratio, leading to reopening of the $K_{\text{ATP}}$ channel, membrane repolarisation, arrest of $\text{Ca}^{2+}$ influx, lowering of $[\text{Ca}^{2+}]_i$, and restoration of a high ATP/ADP ratio to initiate a new cycle. Abbreviation: $\Psi$ - membrane potential of β-cell.
repolarisation, inactivation of voltage-gated Ca\(^{2+}\) channels, reduction in [Ca\(^{2+}\)]\(_i\) and eventual restoration of a high ATP/ADP ratio to initiate a new cycle.

However, data exist that challenges the role of [Ca\(^{2+}\)]\(_i\) oscillations as regulator of oscillatory insulin secretion which argue for the role of \(\beta\)-cell metabolism as the pacemaker. Oscillatory insulin secretion has been observed in these situations: (a) low and non-oscillatory [Ca\(^{2+}\)]\(_i\), resulting from islets being exposed to non-stimulatory glucose concentration level (76); (b) sustained, elevated and non-oscillatory [Ca\(^{2+}\)]\(_i\), when islets were exposed to sustained depolarisation by high K\(^+\) concentration or tolbutamide (77) and (c) \(\beta\)-cell membrane potential is ‘clamped’ with exposure of islets to diazoxide (76,78). Furthermore, oscillatory K\(^+\)\(_{\text{ATP}}\) channel activity has been observed in presence of closed voltage-gated Ca\(^{2+}\) channels suggesting the occurrence of fluctuations in ATP production resulting from glycolytic oscillation (79).

(3) Metabolic hypothesis

The proponents of this hypothesis propose that oscillation of glycolysis in \(\beta\)-cells is the pacemaker for pulsatile insulin secretion. Glycolytic oscillations depend on interaction between glucokinase, phosphofructokinase M isoenzyme and ATP/ADP ratio (80). Following glucose uptake into \(\beta\)-cell, the rapid flux through glucokinase generates ADP and causes a transient decrease in ATP-ADP ratio. This has the dual effect of increasing glycolysis via PFK-M activation and stimulating mitochondrial metabolism via activation of mitochondrial dehydrogenases and the respiratory
chain. This leads to a rise in ATP and ATP/ADP ratio resulting in the closure of the 
$K_{\text{ATP}}^+$ channels and inhibition of glycolysis and mitochondrial respiration until a rise 
in ADP reactivates metabolism and initiates another cycle. Furthermore, 
phosphofructokinase M isoenzyme (PFK-M) which is responsible for generating 
occurrences in glycolysis in skeletal muscle extracts, is also found in β-cell (81). The 
importance of PFK-M is suggested by studies in humans with a genetic deficiency 
of this isoenzyme, in whom oscillations in insulin secretion are impaired (82,83).

Oscillations in glycolysis is a widespread phenomenon and have been shown in 
skeletal muscle extracts (84), yeast cells and extracts (85), heart extracts (86) and 
ascites tumour cells (87). Therefore, it was not surprising to find that glycolytic 
occurrences also occur in pancreatic islets (88) and single β-cells (89). In support of 
the occurrence of glycolytic oscillations in β-cells, the following observations have 
been made; (a) oscillations in lactate release occur with the same periodicity as 
insulin secretion in glucose-stimulated pancreatic islets (88), (b) oscillations of 
similar frequency in O$_2$ consumption, [Ca$^{2+}$]$_i$ and insulin secretion have been 
observed following glucose stimulation of islets (90), (c) Oscillations in NADH 
have been observed in cell-free islet extracts exposed to glucose and glycolytic 
cofactors (91) and (d) oscillations in ATP/ADP ratio (92) and NADPH (74) have 
been demonstrated in glucose-stimulated islets.

It has been argued that oscillations in [Ca$^{2+}$]$_i$ are not necessarily driven by 
occurrences in β-cell metabolism. Since Ca$^{2+}$ can activate mitochondrial
dehydrogenase enzymes to stimulate mitochondrial respiration (74), it was proposed that metabolic oscillations is the consequence of rather than the cause of oscillations in [Ca^{2+}]_{i}. However, it was shown that the rise in NADPH (89) and ATP/ADP ratio (92) occur before the rise in [Ca^{2+}]_{i}, implying that changes in [Ca^{2+}]_{i} is secondary to glucose metabolism. Furthermore, glucose-stimulated rise in ATP/ADP ratio was not prevented by Ca^{2+} channel blockers that prevent the rise in [Ca^{2+}]_{i} (93). In conditions of non-stimulatory glucose concentration where [Ca^{2+}]_{i} is low without the stimulated entry of extracellular Ca^{2+}, cyclic variations in the K_{ATP} channel activity has been demonstrated implying that [Ca^{2+}]_{i} oscillations have a metabolic origin (76).

However, the argument against the metabolic hypothesis was shown by the lack of oscillations in NADPH following stimulation of mouse islets with glucose whilst synchronous oscillations occur in both [Ca^{2+}]_{i} and membrane potential (94). Since NADPH is a reflection of β-cell metabolism and its pattern of rise was monophasic and non-oscillatory, it was felt that the changes in [Ca^{2+}]_{i} and membrane potential is unlikely to result from oscillations in glucose metabolism.

(4) Integration of the ganglia, membrane and metabolic hypothesis
An alternative explanation to account for the pacemaker activity generating pulsatile insulin release is that both glycolysis and membrane potential oscillations are important and that in vivo, they are inter-dependent. Indeed, it would be hard to imagine how these oscillations could be distinct. β-cell metabolism can drive oscillations in membrane potential and [Ca^{2+}]_{i}. Alternatively, oscillations in
membrane potential and \([\text{Ca}^{2+}]_j\) can drive oscillations in \(\beta\)-cell metabolism via the influence of \(\text{Ca}^{2+}\) on mitochondrial dehydrogenases and through the subsequent changes in ATP/ADP ratio on glycolysis and mitochondrial metabolism. It is clear that both metabolic and membrane oscillations are intimately linked to each other. Therefore, it is possible that both oscillate in synchrony to augment and optimise the efficacy of the machinery involved in insulin secretion.

What is the role of intrapancreatic ganglia? Although it may not be necessary for the generation of insulin secretion, it may play a role in fine tuning the \(\beta\)-cell oscillatory behaviour via input from the nervous system. This is supported by the observation that the period of insulin oscillations was altered following truncal vagotomy (95). Furthermore, it can facilitate the spread of electrical activity between pancreatic islets which is required for insulin secretion to occur. In conclusion, it is conceivable that membrane, metabolic and intrapancreatic ganglia are involved simultaneously in the generation and regulation of the pulsatility of insulin secretion.

**Synchronisation of oscillatory insulin secretion**

There are around one million islets in a human pancreas and each islet contains about 2500 \(\beta\) cells (96). Yet, oscillatory insulin secretion is co-ordinated both *in vivo* and *in vitro*. Therefore, there is a mechanism where the spread of electrical activity in the form of depolarisation wave is synchronised throughout the one million islets.

In an intact pancreas, intrapancreatic neural network may play an important role in synchronising the transmission of the electrical wave of depolarisation. This
argument gains support from studies with transplanted islets showing islet to islet innervation is required to achieve co-ordinate pulsatile insulin secretion (97). That extrinsic neural input does not appear to be important in coordinating oscillatory insulin secretion is based upon the observation that patients with a transplanted pancreas have pulsatile insulin secretion (98,99). Furthermore, isolated perfused pancreas preparation without any extrinsic neural network secreted insulin in pulsatile fashion (10).

Groups of perifused islets (without interconnecting nerve fibers) also showed pulsatile insulin secretion (11,12). It has been postulated that a diffusible factor secreted by the islets might co-ordinate the synchronisation process. Various substances have been proposed such as ATP, lactate, pyruvate and insulin but have been proven to be negative (100). At present, the diffusible substance remains unknown and its hypothesis unproven. Alternatively, electrical coupling of several adjacent islets through the perfusion barrier may synchronise the secretion since the \( \beta \)-cell is an electrically excitable tissue and generates electrical current. Careful inspection of insulin release by perifused islets shows that only \(~50\%\) of insulin (or less) (12) is released in co-ordinate pulses whilst single islets release \(~100\%\) of insulin in pulses (101,102). This observation suggests that synchronisation is far from complete in these preparation.

Pulsatile insulin secretion is also observed in perifused single pancreatic islets (101,102). It is known that integrated secretory response of intact pancreatic islet is greater than that of dispersed islet cells and that reaggregation improves secretory
responsiveness suggesting that intra-islet interactions are essential for normal secretory responses (103-106). The nature of these intra-islet interactions remains uncertain. It has been proposed that the paracrine effects of glucagon (106) or glucagon-like peptide 1 (107) which act through the cAMP-dependent protein kinase A (PKA) pathway might be responsible. However, the paracrine effects may not have a major influence on islet cell function since inhibition of PKA pathway in intact islets did not affect basal or glucose-induced insulin secretion (108,109). Direct contact between β-cells appears to be necessary to enhance nutrient-induced insulin secretion (105,110). Within an islet, β-cells are connected by gap junctions composed of the protein connexin 43 (Cx43) (111) and are electrically coupled (112). This raises the possibility that gap junction communication between β-cells may play a role in co-ordinating the heterogeneous response of individual β-cells to produce the observed integrated secretory response of the whole islet (113). This important role of gap junction is further supported by the following observations; (a) gap junction blocker, heptanol, has been shown to abolish glucose-induced insulin secretion from intact islets but not from single β cells (114); (b) gap junctions and coupling increase between β-cells during sustained stimulation of insulin secretion (115) and (c) isolated β-cells showed impaired secretion which improved after re-establishment of gap junctions (116). Apart from communication between β-cells, interaction between β and non-β cells within the microenvironment of the islet appears to be important also (117).
Taking a holistic approach, one could envisage the initiation of the electrical wave of depolarisation from the β-cells and/or intra-pancreatic ganglia with its subsequent transmission through each individual β-cell via gap junctions with concomitant interaction occurring between β-cells and non-β cells and subsequently involving the adjacent and distant islets via the intra-pancreatic neural network in the process of electrical coupling of the whole pancreas to create a co-ordinated secretory burst with its frequency being determined by the pancreatic pacemaker which is modulated, perhaps, by external influence such as extrinsic pancreatic neural connections.

**Regulation of insulin secretion**

Since insulin is secreted in discrete bursts, establishing an accurate method for the detection and quantification of insulin secretory bursts is crucial to the understanding of the regulation of pulsatile insulin secretion. Quantification of the pulsatile insulin secretion presents several difficulties. Firstly, the frequency of insulin secretory bursts (pulse interval ~ 5-8 mins) is several-fold greater than that of the more extensively studied hypothalamo-pituitary hormones (pulse interval ~ 60 mins). Secondly, insulin is secreted into the portal circulation and undergoes significant hepatic extraction and waveform damping before entry into the systemic circulation. Thirdly, whereas there are well-established methods available to quantify secretion rate of the pituitary hormone (118), there are no validated methods to quantify pulsatile insulin secretion. However, these difficulties were recently overcome by employing a canine model in which pulsatile insulin secretion was measured directly in the portal vein by catheterisation across the pancreas (8).
This study provided the opportunity to validate an established deconvolution program for the measurement of pulsatile insulin secretion and applied this technique to quantify pulsatile insulin secretion from peripheral blood measurements. Subsequently, this method of deconvolution analysis was validated for the quantification of pulsatile insulin secretion from peripheral blood measurements in human beings (15).

By employing the validated deconvolution method to quantify pulsatile insulin secretion in vivo, it was subsequently found that majority of insulin was secreted in the pulsatile component (at least 70%) in the fasting phase (8,15). Furthermore, the regulation of insulin secretion occurred via modulation of the pulsatile component predominantly through regulation of its secretory burst mass. For example, in a canine model, the increment in insulin secretion after glucose ingestion was achieved by a ~400% increase in insulin secretory burst mass and ~40% increase in burst frequency (16). Furthermore, other studies showed that inhibition of insulin secretion via somatostatin (in dogs) (17) and IGF-1 (in humans) (119) and conversely, stimulation of insulin secretion via GLP-1 (in humans) (19) and sulphonylurea (in dogs) (18), were achieved predominantly by inhibition and amplification of insulin secretory burst mass respectively. Although deconvolution program has yet to be validated for the detection and quantification of pulsatile insulin secretion from perifused pancreatic islets, studies have shown that perifused groups of islets (90) and single islets (101) exposed to elevated levels of glucose augment their insulin secretion via amplification of the pulse amplitude. This raised
the possibility that, at the level of pancreatic islets, the regulation of insulin secretion is most likely to occur through the modulation of its pulsatile component.

Since the regulation of insulin secretion is predominantly achieved by changes in its secretory burst mass, the question arises, what is the mechanism by which the burst mass is altered? Insulin secretory burst mass may be regulated by the number of \( \beta \)-cells per islet recruited per burst and/or the size of the immediately releasable insulin pool secreted by each \( \beta \)-cell per burst. The former may occur via recruitment of \( \beta \)-cells that are quiescent at low glucose levels in conditions of raised glucose concentration (90). This model was proposed by Pipeleers et al (120) who reported that there is heterogeneity in glucose sensitivity at different glucose concentration level among the \( \beta \)-cells. Alternatively, the size of immediately releasable insulin pool can be modulated. GLP-1 is thought to act via this mechanism by increasing the sensitivity of \( \beta \)-cells to the prevailing intracellular ATP concentrations resulting in amplification of insulin secretion from immediately releasable insulin pool following depolarisation (19). Sulphonylurea is also thought to act similarly by increasing the proportion of pre-existing insulin granules that undergo exocytosis (18).

Whilst the frequency of insulin secretory burst remains relatively unchanged as opposed to the pulse mass or amplitude (16-19,68), it implies that the intrinsic rhythm of the pancreatic pacemaker which generates pulsatile insulin secretion is
resistant to both stimulatory and inhibitory stimuli. Thus, the intra-pancreatic pacemaker activity is a remarkably stable phenomenon.

**Physiological relevance in health**

In humans, the pituitary hormones are secreted in a pulsatile manner (1,2). Since the pancreas forms part of the endocrine system, it is not surprising that insulin is secreted in pulsatile manner also. The biological importance of this inherent pattern of secretion is to prevent receptor down-regulation and thus, enhance the hormone effect on target tissues. Insulin receptors have been shown to down-regulate when exposed to high steady-state insulin concentrations *in vitro* (121) and *in vivo* (122). The greater hypoglycaemic effect of pulsatile insulin delivery in human subjects was first shown by Matthews et al (123). Subsequently, this observation was confirmed by other studies and the hypoglycaemic action was mediated through suppression of hepatic glucose release (124-126). In these studies, insulin was infused into the peripheral circulation.

As insulin is secreted directly into the portal vein, it is plausible that it acts directly to suppress hepatic glucose production (127). Recently, this direct inhibitory action of insulin on hepatic glucose production was demonstrated in human beings (128). Hepatic insulin action and hepatic insulin clearance are mediated through binding of insulin to its receptor on hepatocyte plasma membrane (129). Following the binding of insulin to its hepatic receptor, the complex of bound insulin and its receptor is internalised. Intracellular signalling is generated and initiates the action of insulin on glucose metabolism and insulin clearance within the liver. The receptor is then
recycled and reinserted into the hepatocyte plasma membrane. The duration of this receptor recycling is reported to be 5-10 minutes (130). This is similar to the physiological range of insulin pulse frequency, thus, maintaining the optimal number of the hepatic insulin receptors to bind with the insulin molecules arriving as pulses. In addition, pulse amplitude plays an important role in determining hepatic sensitivity to insulin action. There is a correlation between the pulse amplitude and hepatic insulin clearance (17) and the amount of hepatic insulin receptors bound and internalised (130). It is plausible that hepatocytes preferentially bind insulin delivered in pulses that create a high peak concentration typically seen within the portal circulation.

Pulsatile insulin secretion has been shown to be abnormal in type 2 diabetes subjects (20). Since the magnitude of insulin pulses in the peripheral circulation is attenuated in this condition, it is likely that the liver in these patients is exposed to even more attenuated oscillations in insulin pulse amplitude. It is plausible that the diminished insulin pulses in the portal circulation may contribute to the hepatic insulin resistance and excessive hepatic glucose production, the inherent features of type 2 diabetes mellitus (131). Support for this notion is derived from the observation that hepatic clearance of portal vein insulin is related to the pulse amplitude of the insulin concentration wavefront presented to the liver in a canine model (17). Reduction in the number of insulin molecules bound to their hepatic receptors and internalised to exert its biological function could be the mechanism. However, the direct effect of portal insulin pulse amplitude modulation on hepatic glucose release in human beings remains to be studied.
**Potential causes for abnormal oscillatory insulin secretion**

Many potential causes for the impaired pulsatile insulin secretion have been proposed. One possible cause is loss of pacemaker function. However, the presence of pulsatile insulin secretion in patients with type 2 diabetes mellitus (20) and in groups of perifused islets from these patients *in vitro* (132) argue against this point. Alternatively, defects in intra- or interislet communication system may account for the abnormal oscillatory insulin secretion. Defects in intraislet communication between β-cells may be caused by deposition of islet amyloid between β-cells resulting in disruption of gap junction communication which play important role in synchronising insulin secretion (133). Deposition of cytotoxic human amyloid can be enhanced by the increased rate of amyloid secretion induced by insulin resistance or decreased availability and/or function of heat-shock proteins (133). Recently, it has been shown that cytotoxic human amyloid induce β-cell death by apoptosis and pore formation in the cell membrane (134,135). It is conceivable that loss of β-cells, especially the subpopulation of ‘pacemaker’ cells, can lead to disruption of the oscillatory secretion. Another potential cause is the disruption of intra-pancreatic neural network. Although autonomic neuropathy is a well-known late complication of diabetes (136), there is no evidence this is present prior to the onset of clinical diabetes when the abnormal oscillatory insulin secretion is already present. Therefore, it is unlikely that this factor play a mechanistically important role in the early stages of type 2 DM. Another potential cause is the depletion of immediately releasable pool of insulin so that when a wave of depolarisation reaches the β-cell, there is sub-optimal mass of insulin available to be discharged. Recently, it has been
shown that the pool of immediately releasable insulin is responsible for the first phase insulin secretion (137). The observation of diminished first phase insulin secretion in type 2 diabetes mellitus (138) supports the hypothesis of depleted insulin pool. This hypothesis of impaired insulin secretion as a consequence of depleted insulin stores is one of the issues addressed in this MD thesis and will be discussed in more detail in the following section.

**Effect of β-cell rest on insulin secretion**

Pancreatic β-cell mass is reduced by at least 50-60% in type 2 diabetes mellitus (139-141). Hence, it is plausible that the reduction in β-cell mass (and insulin stores) might contribute to defective insulin secretion in type 2 diabetes. Following reduction in β-cell mass, there is an increased secretory demand on the remaining β-cells to compensate for this loss. This enhanced β-cell secretory function is further augmented by the presence of peripheral insulin resistance and hyperglycaemia. Eventually, this state of chronic over-stimulation of β-cell secretory function exceeds its insulin synthesis capacity leading to depletion of insulin stores resulting in impaired glucose-induced insulin secretion.

Most of the evidence to support this hypothesis came from animal studies. Rat islets exposed to high glucose concentrations had impaired insulin secretion and reduced insulin stores (142). Surgically-reduced β-cell mass as a model of type 2 diabetes i.e. 90% pancreatectomised Sprague-Dawley rats developed hyperglycaemia which was associated with impaired insulin secretion and depleted insulin stores (143). Normal
Sprague-Dawley rats exposed to tolbutamide to chronically stimulate insulin secretion in presence of normoglycaemia also led to depletion of insulin stores and impaired insulin secretion (144). In humans, healthy non-diabetic subjects who underwent hemipancreatectomy for the purpose of organ donation, had raised fasting plasma glucose, impaired insulin secretion, abnormal glucose tolerance and reduced maximal acute insulin response (AIR_max) (145,146). Irrespective of the mode of stimulation of insulin secretion (whether it is hyperglycaemia or non-hyperglycaemia-induced), the end result was similar; impaired glucose-induced insulin secretion with depleted insulin content.

Further evidence to support this concept came from studies looking into the cause of relative and absolute hyperproinsulinaemia in Type 2 diabetes. Metabolic clearance of proinsulin is unchanged in subjects with type 2 diabetes (147,148), indicating that hypersecretion of proinsulin accounts for the raised plasma proinsulin levels (149,150). Two hypotheses were proposed to explain the aetiology for the hyperproinsulinaemia. First, a defect in the enzymatic proinsulin processing mechanism within the β-cell was suggested (147). This defect could occur in the post-translational modification of proinsulin molecule leading to proinsulin-rich granule. Alternatively, a heightened demand on insulin secretion from hyperglycaemia can deplete the β-cell of mature granules, resulting in an enriched population of immature (proinsulin-rich) granules for secretion (151). In a study by Alarcon et al, it was shown that normal rats made hyperglycaemic with 48-hr glucose infusions had a raised percentage of pancreatic proinsulin to insulin ratio (151). The pancreatic insulin content was reduced whilst the proinsulin content
remained unchanged. Moreover, there was a decrease in the islet content of proinsulin conversion enzymes, PC2, PC3 and CP-H. It was observed that the release of PC2, PC3 and CP-H enzymes was increased along with insulin and there was no associated deficiency in the biosynthesis of PC2, PC3, CP-H and proinsulin. Proinsulin conversion rate remained unaffected by the hyperglycaemic condition. These observations suggested that hyperproinsulinaemia induced by hyperglycaemia is likely to result from increased β-cell demand rather than a defect in the proinsulin processing enzyme.

If this concept of chronic over-stimulation of β-cell secretion with subsequent defective glucose-induced insulin secretion is valid, then ‘resting’ the pancreas by lowering its insulin secretory demand should result in improved endogenous insulin secretion and content. β-cell rest can be achieved by two ways; (1) indirectly by reducing the glucose load on the pancreas through normalisation of the plasma glucose concentration, (2) directly by pharmacologically-induced inhibition of β-cell insulin secretion by agents such as diazoxide or somatostatin. There is evidence from both human and animal models that these two methods of β-cell rest lead to improved insulin secretion. Normalising plasma glucose in type 2 diabetic subjects by intensive insulin treatment led to improvement of first (152) and second (153) phase insulin secretion. Furthermore, treatment by diet and sulphonylurea medication to achieve normal glucose level also led to improvement in endogenous insulin secretion in subjects with Type 2 diabetes (154,155). Hales et al (156) administered oral diazoxide to subjects with Type 2 diabetes for 5 days and showed
improvement in first phase insulin secretion. This observation was supported by a study by Laedtke et al (157) who infused somatostatin overnight to ‘rest’ the β-cells which subsequently resulted in the restoration of first phase and pulsatile insulin secretion. However, these human studies do not demonstrate the effect of β-cell rest on the pancreatic insulin content. This question was addressed by Leahy et al (143) who administered diazoxide to a surgically-reduced β-cell mass model of type 2 diabetes i.e. 90% pancreatostomised Sprague-Dawley rats. This study showed that the enhancement in pancreatic insulin secretion was associated with an increment in pancreatic insulin stores, normalisation of plasma glucose concentration and oral glucose tolerance demonstrating the beneficial effects of β-cell rest on its function was mediated by restoration of pancreatic insulin content.

Further support for the concept of β-cell dysfunction as a consequence of hyperstimulation came from studies that studied the effects of β-cell rest on hyperproinsulinaemia in hyperglycaemic condition. In normal rats made hyperglycaemic by glucose infusion, diazoxide resulted in the reduction of the pancreatic proinsulin/insulin ratio by attenuating the fall of pancreatic insulin content leading to greater quantity of mature (insulin-rich) granules (150). In human islets exposed to high glucose concentration, diazoxide also reduced the proinsulin/insulin ratio and increased the islet insulin content (158). In subjects with type 2 diabetes, proinsulin/insulin ratio was normalised in conditions of euglycaemia achieved by diet or sulphonylurea medication (154).
The working model of chronic stimulation leading to β cell dysfunction and impaired insulin secretion is depicted in figure 6. With the reduction in β cell mass, hyperglycaemia ensues. This stimulates the residual β cells to secrete insulin excessively, perhaps, by increasing β cell sensitivity via upregulated activity of hexokinase (159) or hyperactivation of the entire β cell population with the loss of the intercellular heterogeneity (142). Excessive insulin secretion outstrips insulin synthesis leading to reduction in insulin stores, mediating two functional consequences: reduced insulin secretion and relative hyperproinsulinaemia (raised proinsulin to insulin ratio).

**MD projects**

From the discussion above, four related projects were performed to address the pattern of insulin secretion and the specific mechanism by which β-cell positively influences these:

**Project one:**
Direct measurement of the pattern of insulin release in the portal vein in human subjects in the basal state and in response to glucose stimulation. This will be achieved by using the validated method of deconvolution to analyse the pattern of insulin secretion.

**Project two:**
Examination of the effects of acute inhibition of insulin secretion by diazoxide on the pattern of insulin secretion in patients with Type 2 diabetes mellitus.
Hyperstimulation of insulin secretion

Insulin resistance

↑ sensitivity of β-cells

↑ hexokinase activity

? hyperactivation of β-cells from loss of intercellular heterogeneity

Reduced insulin stores

Reduced insulin secretion

↑ proinsulin secretion

↑ proinsulin/insulin ratio

Figure 6

Pathophysiology of impaired glucose-induced insulin secretion from the loss of β-cell mass.
Project three:
Development and validation of a method to quantify pulsatile insulin secretion by cultured human pancreatic islets in the perifusion system.

Project four:
Application of the system developed and validated in project three to test the hypothesis that transient β-cell rest in human pancreatic islets induced by diazoxide attenuates the loss of first phase, second phase and pulsatile insulin secretion caused by culture of the islets in glucose concentration comparable to those seen in Type 2 diabetes and to examine the effect on islet insulin content on insulin release.

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Chapter 2

Direct measurement of pulsatile insulin secretion from the portal vein in human subjects
Introduction

Insulin is secreted in high-frequency pulses (1). This pattern of insulin secretion has been shown to be abnormal in subjects with Type 2 diabetes mellitus (2) and their first-degree relatives (3) and patients at risk of developing Type 1 diabetes (4). However, quantification of pulsatile insulin secretion is complex. Insulin pulses are secreted into the portal circulation and undergo significant hepatic extraction and waveform damping before entering the systemic circulation (5). We previously developed a canine model of direct portal vein catheterisation to overcome this problem (6), and reported that sampling from the systemic circulation resulted in an underestimate of both the frequency of insulin pulses as well as the calculated proportion of insulin released in the pulsatile mode (5). Inevitably studies of pulsatile secretion in humans have been confined to the systemic circulation (2-4,7-12). Initially such studies reported a pulse frequency of 15-20 minutes (2-4,7,8) but more recently a pulse frequency of ~6 minutes has been suggested (11,12). We have speculated that these differences may reflect the greater sensitivity of novel insulin assays applied in the latter studies, and that the true frequency of insulin pulses delivered into the portal circulation in humans may be 6-8 minutes. The latter would be comparable to the frequency observed in the isolated perfused pancreas (13) as well as the perifused islets (14-15).

In the current study, we applied a validated deconvolution technique for quantifying pulsatile insulin secretion to plasma insulin concentration profiles obtained simultaneously from the portal vein and the systemic circulation in human subjects with stable compensated hepatic cirrhosis and an in situ transjugular intrahepatic portasystemic stent shunt (TIPSS) (16). The TIPSS catheter allowed relatively noninvasive high-frequency sampling of blood
from the portal vein in conscious human subjects. Pulsatile insulin secretion was quantified in the fasting basal state as well as during a hyperglycemic clamp study.

Using this protocol we sought to address the following questions. First, is the frequency of pulsatile insulin secretion in humans (observed by direct sampling from the portal circulation) comparable with the frequency reported previously from studies in isolated islets, the isolated pancreas and in vivo by sampling from the portal vein in dogs (interpulse interval ~6-8 minutes) or ~15-20 minutes as reported in some studies using the systemic sampling site in humans? Second, does insulin secretion in humans match the dynamics reported in dogs based on direct portal vein sampling, wherein the majority of insulin is secreted in discrete bursts? And third, does hyperglycemia in the human (as in the dog) enhance insulin secretion through the specific mechanism of augmenting the mass of insulin bursts? Finally, we examined whether the amplitude of the insulin-concentration wavefront to which the liver is exposed in humans approaches that observed in the portal vein of dogs (5,6).

**Methods and material**

**Study subjects and design** (tables 1 and 2)

This study was approved by the Lothian Ethics Committee and all volunteers provided informed written consent. Inclusion criteria were patients with a patent TIPSS and known stable compensated liver disease. Patients were deliberately selected to represent those in which the predominant liver-related problem was cirrhosis and portal vein hypertension treated with a transjugular intrahepatic portosystemic stent shunt (TIPSS), rather than progressive liver failure or active hepatic inflammation. Exclusion criteria include
Table 1: Patient characteristics

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
<th>FPG (3.9 – 6.1) mmol/L</th>
<th>Glucose tolerance</th>
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<tr>
<td>1</td>
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<td>F</td>
<td>60</td>
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<tr>
<td>2</td>
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<td>M</td>
<td>71</td>
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<tr>
<td>3</td>
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<td>M</td>
<td>71</td>
<td>24</td>
<td>4</td>
<td>Impaired</td>
</tr>
<tr>
<td>4</td>
<td>61</td>
<td>M</td>
<td>100</td>
<td>33</td>
<td>5.8</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>54</td>
<td>M</td>
<td>117</td>
<td>36</td>
<td>5.4</td>
<td>Impaired</td>
</tr>
</tbody>
</table>

Legend

FPG= fasting plasma glucose; M= male; F= female, BMI = body mass index

Table 2: Liver function studies

<table>
<thead>
<tr>
<th>Type of Cirrhosis</th>
<th>Alk phos (40-125 u/L)</th>
<th>GGT (5-35 u/L)</th>
<th>Bilirubin (2-17 uM)</th>
<th>Albumin (36-47 g/L)</th>
<th>ALT (10-40 uL)</th>
<th>INR (2-4.5)</th>
</tr>
</thead>
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<td>124</td>
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<td>43</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
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<td>100</td>
<td>42</td>
<td>34</td>
<td>56</td>
<td>1.3</td>
</tr>
<tr>
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<td>61</td>
<td>147</td>
<td>35</td>
<td>35</td>
<td>1.7</td>
</tr>
<tr>
<td>alcoholic</td>
<td>92</td>
<td>54</td>
<td>17</td>
<td>41</td>
<td>33</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Legend

ALT alanine amino transferase
Alk Phos alkaline phosphatase
GGT gamma-glutamyl trasferase
INR International Normalized Ratio
decompensated liver dysfunction, diabetes mellitus, a thrombosed stent, and/or a prolonged prothrombin time. All patients were studied 1-2 weeks prior to the TIPSS study to have a 75 gram oral glucose tolerance test performed to exclude diabetes mellitus. Of six subjects screened, one was excluded from the study because of diabetes.

Five patients with in-situ TIPSS were studied immediately after a routine follow-up assessment of the patency of their TIPSS. For this purpose patients were admitted overnight into the Royal Infirmary of Edinburgh and after an overnight fast a catheter placed in the right jugular vein and under ultrasound guidance passed through the TIPSS into the portal circulation. The purpose of the clinical assessment was to ensure that the TIPSS remained patent. Once this had been confirmed, a catheter was inserted via the right internal jugular introducer sheath into the portal vein to allow the present study to be completed.

Study protocol

All study subjects were admitted to the University of Edinburgh Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh the night prior to the study and remained fasting overnight. On the morning of the study, each subject underwent TIPSS portogram performed by a radiologist as part of the routine follow-up assessment of the patency of the in-situ TIPSS and the sampling catheter placed in the portal vein at the end of this procedure.

A peripheral indwelling sampling catheter was also inserted into a dorsal hand vein and the hand was warmed to 40°C by an electric blanket to permit subsequent sampling of arterialized blood. An intravenous catheter was also placed in an antecubital vein in the
contralateral arm and infused with saline at 30 ml/hour. Once all the catheters were in place there was a 45 minute rest period prior to commencement of the protocol at ~10:00 am. At protocol time 0-40 minutes, simultaneous intensive 1-minute sampling of arterialized blood and portal vein blood were performed to obtain the minutely insulin concentration profile at each site in the fasting state. At protocol time 40 min, a hyperglycaemic clamp was commenced with the object of raising the arterialized plasma glucose concentration to 8-9 mmol/l. This was achieved by infusion of a variable rate glucose infusion (Dextrose 50%) administered by a programmable infusion pump (Harvard Infusion Pumps, Ayer, MA) on-line to a personal computer. Arterialized blood was sampled at 5-minute intervals and the plasma glucose measured within 2 minutes. Steady state hyperglycemia was achieved by protocol time 80 minutes (40 minutes after the clamp was begun) and then the second sampling period began. From protocol time 80-120 minutes, blood was sampled at one-minute intervals from the arterialized peripheral vein catheter and the portal vein catheter to determine the insulin concentration profile during the hyperglycemic clamp. Portal vein sampling was completed in all 5 study subjects in both the basal and hyperglycemic sampling periods. Arterialized sampling was completed in four of the five subjects, the peripheral sampling catheter was unreliable in the remaining subject. In two subjects portal vein blood flow was measured during the study by use of Doppler scanning.

**Assays**

*Glucose.* Plasma glucose concentrations were measured by the glucose oxidase method using a Beckman glucose analyser (Beckman, Palo Alto, CA).
Insulin. Plasma insulin concentrations were measured in duplicate by two-site immunospecific insulin ELISA, as previously described (11,17). In brief, the assay uses two monoclonal murine antibodies (Novo Nordisk, Bagsvaerd, Denmark) specific for human insulin. The detection range of this insulin ELISA was 5-600 pmol/l. At medium (150 pmol/l), medium-high (200 pmol/l) and high (350 pmol/l) plasma insulin concentrations, the interassay coefficients of variation were 3.7%, 4.0%, 4.5% and the corresponding intra-assay variations were 2.3%, 2.1%, 2.0%. There was no cross-reactivity with proinsulin and split 32,33 and des 31,32 proinsulins, respectively.

C-peptide. Plasma C-peptide concentration was assayed with a commercially available kit (K6218; DAKO, Cambridgeshire, UK). The assay is a two-site ELISA based on two monoclonal antibodies, using the same principles referred to above. Each sample was assayed in duplicate; intra- and inter-assay variation coefficients were 2.2% and 3.3% respectively.

Data analysis

Detection and quantification of pulsatile insulin secretion by deconvolution analysis.

The plasma insulin concentration time series were analysed by deconvolution as previously validated (18) to detect and quantify insulin secretory bursts (6). Deconvolution of plasma insulin concentration data was performed with a multiparameter technique that requires the following assumptions. Plasma insulin concentrations measured in samples collected at 1-min intervals were assumed to result from five determinable and correlated parameters: 1) a finite number of discrete insulin secretory bursts occurring at either regular or randomly dispersed times and having a) individual amplitudes (maximal rate of secretion attained
within a burst) and mass (integral of the calculated secretory event) and b) a common half-duration (duration of an algebraically Gaussian secretory pulse at half-maximal amplitude), which are superimposed on c) a basal time-invariant insulin secretory rate; 2) a bi-exponential insulin disappearance model in the systemic circulation, consisting of earlier directly estimated half-lives of 2.8 and 5.0 min and a fractional slow component of 0.28 in healthy fasting humans; 3) a bi-exponential insulin disappearance model in portal circulation, consisting of half-lives of 1.0 and 3.0 min and a fraction slow component of 0.667. These parameters achieved the statistically best fit (maximally reduced fitted variance) of the portal vein insulin concentration profile. All secretory rates were expressed as mass units of insulin (picomoles) released per unit distribution volume (liters) per unit time (minutes).

Statistical analysis

Data are presented as the mean ± SEM. Statistical comparison between groups was made by Student’s two-tailed t-test. In order to examine the relationship between insulin concentration fluctuations observed in the portal vein and systemic circulation, cross correlation was performed. Cross correlation analysis relates each portal vein insulin concentration to a corresponding value in the matching arterial series. This procedure consists of linear correlations carried out repeatedly at various time lags between the paired concentrations. Thus, at zero time lag, each portal vein plasma insulin concentration is compared with a time delayed measure (e.g. lag time minus 2 minutes) in the systemic circulation sample. By this means, one collects an array of correlations that depend on the time matching of the two series.
In order to examine the relationship between pulses identified in the portal vein and systemic circulation we also performed peak concordance on these pulses from both sites. Peak concordance is a statistically independent (from cross correlation) method to establish the relationship between detected pulses in two sampling sites. After identifying discrete insulin secretory bursts in the two time series (portal and peripheral) exact coincidence was defined by simultaneous pulse concordance (i.e. peak maxima occurred within one-half sampling interval of each other). Lagged coincidence was defined accordingly (e.g. with portal (+lags) or peripheral (-lags) peaks occurring first. The hypergeometric probability density (joint binomial distribution) was used to estimate the expected number of randomly concordant pulses, and the probability of falsely refuting the null hypothesis of pure chance concordance of the observed coincidences.

**Results**

*Mean plasma glucose, insulin and C-peptide concentrations* (figure 1)

The mean arterialized plasma glucose concentrations during the basal (fasting) and stimulated (hyperglycemic clamp) sampling periods were 5.3±0.3 and 8.1±0.1 mmol/l respectively (p<0.001). As expected, there was a rise in the mean arterialized (basal vs stimulated, 209±7.4 vs 456±16.8 pmol/l, p<0.001) and mean portal vein (basal vs stimulated, 440±25.3 vs 1020.7±72.3 pmol/l, p<0.001) insulin concentration following the increase in the plasma glucose concentration from 5.3 to 8 mmol/l during the hyperglycemic clamp. The mean arterialized C-peptide concentration also increased with hyperglycemia (basal vs stimulated, 1.8±0.1 vs 2.7±0.1 nmol/l, p<0.001) confirming an increase in insulin secretion in response to the glucose stimulus. Throughout the study in each subject, the
Mean arterialised plasma glucose (top panel), C-peptide (middle panel) and both arterialised (open squares) and portal vein (solid squares) insulin concentrations. T=0-40 min corresponds to the basal intensive sampling period. The hyperglycaemic clamp was initiated at t=40 min and steady state hyperglycaemia was achieved by t=80 mins when the second intensive sampling period was undertaken.
portal vein insulin concentration was higher than the corresponding arterialized insulin concentration in both basal (p<0.001) and stimulated (p<0.001) sampling periods (figure 1).

*Portal vein blood flow*

The mean portal vein blood flow was in the 2 cases in which it was measured 1.1 L/min and 0.8 L/min. There was no change in the portal vein blood flow between the basal state and the hyperglycemic clamp.

*Insulin concentration profile* (figure 2, 3)

Inspection of the plasma insulin concentration profiles from the individual patients indicated the presence of recurrent oscillations in both arterial and portal circulation in all patients. The magnitude of these oscillations was much larger in the portal circulation (figure 2) in both the basal and stimulated states compared with the corresponding profiles from the systemic circulation. The amplitude of the oscillations increased during hyperglycemia (figure 2). The range of the insulin pulse amplitude observed in the portal circulation was 100–1000 pmol/l in the basal state and increased to 200-3000 pmol/l during the hyperglycemic clamp. This contrasts with the corresponding range of pulse amplitudes observed in the systemic circulation of 10-30 pmol/l and 40-100 pmol/l in the basal and stimulated states respectively (figure 3).

*Pulse detection*

When the insulin concentration profiles were subjected to deconvolution, insulin pulses were invariably identified in both the portal and systemic circulation (figures 4a,4b,5a,5b). The pulse mass increased in response to hyperglycemia in both portal (basal vs stimulated,
Fig 2.

Plasma insulin concentration profiles from the basal (t=0-40 min) and hyperglycaemic clamp (t=80-120 min) period in 2 cases obtained from the arterialised (open squares) and portal vein (solid squares) sampling sites. The scales are adjusted in the left and right panels to accommodate the insulin concentration range observed. Oscillations in insulin concentrations are much greater in the portal vein than the arterialised sampling site.
Case 2

Fig 3.

Plasma insulin concentration profile observed from the arterialised sampling catheter in case 2 in the basal state (t=0-40 min) and during hyperglycaemia (t=80-120 min). The scale has been adjusted for each sampling period to maximise the visualization of oscillations. In comparison, with the arterialised insulin concentration profiles in figure 2, the expanded scale clearly illustrates the prominent oscillations in insulin concentration in both basal and stimulated state.
Fig 4a.
The portal vein insulin concentration profile and corresponding deconvolved insulin secretion rates obtained the intensive sampling periods in the basal state and during hyperglycaemic clamp periods for case 1.
The arterialised insulin concentration profile and corresponding deconvolved insulin secretion rates obtained during the intensive sampling periods in the basal and during hyperglycaemic clamp periods for case 1.
Fig 5a.
The portal vein concentration profile and corresponding insulin secretion rates obtained during the intensive sampling periods in the basal state and during hyperglycaemic clamp periods for case 2.
Arterialized (Case 2)

Fig 5b.
The arterialised insulin concentration profile and corresponding deconvolved insulin secretion rates obtained during the intensive sampling periods in the basal and during the hyperglycaemic clamp periods for case 2.
418±155 vs 1078±368 pmol/l, p<0.05) and systemic (basal vs stimulated, 75±10 vs 241±61 pmol/l, p<0.05) circulation (figure 6). Furthermore, the measured pulse mass/volume of distribution was ~5-fold larger in the portal circulation in both basal and stimulated states. The pulse amplitude determined by deconvolution was also markedly (7-8 fold) larger in the portal circulation in both basal (portal vs systemic, 254±158 vs 23±2.7 pmol/l/min) and stimulated (portal vs systemic, 524±337 vs 72±19.8 pmol/l/min) states confirming the impression gained by inspection of the insulin concentration profiles. The corresponding four pulse half-duration estimates were similar, with a global mean of 2.9 min. The inter-pulse interval (figure 6) determined by direct portal vein sampling was similar in both the basal sampling period as well as during the clamp (figure 6). The pulse interval detected by sampling from the systemic circulation was slightly but not significantly higher than that observed in the portal circulation.

In the portal circulation, the calculated proportion of insulin derived from discrete insulin secretory bursts was 64±1.6% in the basal state and 93±2.9% during the hyperglycemic clamp. In contrast, in sampling from the systemic circulation, the calculated proportion of insulin secreted in the pulsatile mode was apparently lower than from the simultaneous sample measurements obtained from the portal circulation (basal state; portal vs systemic, 66±1.8 vs 35±4.1%, p<0.05) and stimulated (portal vs systemic, 93±2.9 vs 56±1.2%, p<0.01). Both autocorrelation and peak concordant analysis independently revealed a relationship between insulin oscillations in the portal vein and the systemic circulation (figures 7,8).
Fig 6.

The mean insulin inter-pulse interval (top panel), pulse mass (middle panel) and pulse amplitude (bottom panel) during the basal sampling period and the stimulated hyperglycaemic period evaluated by sampling from the portal vein (open panels) versus the arterial catheter (shaded panels). Units are min (pulse interval), pmol/L (mass) and pmol/L/min (amplitude)
Figure 7. Cross-correlation analysis of arterial and portal vein insulin concentration data in the basal and stimulated state. These data illustrate that changes in serial portal vein insulin concentrations precede those in the systemic circulation by 1 to 3 minutes (p<0.05, p<0.01). Line (-) denote the median value.
Figure 8. Peak concordance analysis of pulses detected in the portal vein and systemic circulation. Observed number of coincident insulin secretory bursts detected by simultaneous sampling in the portal and peripheral circulation. The x axis gives the lag interval between the individually identified portal and peripheral insulin pulses. A lag interval of (-2, -1) denotes that pulses occurred centered within this absolute time lag (i.e. the pulses in the portal blood followed those of insulin in the peripheral blood by no less than 1 minute and no more than 2 minutes). Thus, at a lag interval of (+1, +2), insulin pulses in the portal blood preceded those in the peripheral blood by no less than 1 minute and no more than 2 minutes (right side of graph). The expected number of purely random coincidences was calculated via the hypergeometric probability distribution in both the basal and stimulated states (interrupted lines). The observed number of coincidences significantly exceeded expectation values at 1 to 3 minute lag times, wherein portal insulin pulses appeared first. The maximal coincidence was 13 events in the basal state, and 17 events in the stimulated state. The corresponding expected values were 9 and 11 given the different pulse frequencies at these times, with respective P values less than 10^{-5}. 
Discussion

The present study uses direct and high-frequency sampling from the portal vein to affirm that insulin is secreted in humans almost exclusively in discrete secretory bursts, and that hyperglycaemia enhances insulin secretion by selective amplification of the mass of insulin contained within each secretory burst. Furthermore, we could establish that the pre-hepatic frequency of insulin pulses is ~5 minutes, which in fact corresponds with that observed in the portal vein of dogs \textit{in vivo} (5,6), and from isolated perfused islets \textit{in vitro} (14,15).

There has been some disagreement in the literature as to the frequency of pulsatile insulin secretion \textit{in vivo} with estimates ranging from ~6 (5,6) to ~20 (2,3,4,7,8) minutes. When we measured pulsatile insulin release directly from the canine portal vein, the interpulse interval averaged 6 minutes, based on a conventional insulin immunoassay and a deconvolution program specifically validated for insulin pulse detection (5,6). Although this pulse frequency corresponded to that observed in perfusion of single islets \textit{in vitro}, it was three times greater than that observed previously in humans by sampling from the systemic circulation (2-4,7,8). We postulated that the reason for this large discrepancy was the relative loss of insulin signal when insulin pulses are examined in the systemic compared with the portal circulation (6).

At least in the canine model, we confirmed that the sampling site was crucial when a conventional insulin assay was employed (5), since simultaneous estimates in the portal vein and the systemic circulation revealed an insulin interpulse interval of 6.7 minutes and 9 minutes, respectively (5). The insulin pulse detection was further obscured by using a 2-minute sampling regime in the systemic circulation, as employed in some earlier reports.
The disadvantages of sampling from the systemic circulation were especially evident when the only available insulin assays were conventional immunoassays (20). Even multiple replicates by these assays, which have cross reactivity with proinsulin and insulin split products, do not allow for fully reliable detection of high frequency insulin pulses in the systemic circulation. However, the introduction of the more sensitive and specific ELISA assays for insulin (17) should theoretically overcome some of these problems. Recently, using an ELISA for insulin measurements, we reported an insulin pulse frequency of ~8 minutes when sampling in the systemic circulation in humans (11,12). The present study now confirms directly that the systemic sampling route can provide an accurate estimate of insulin pulse frequency in vivo in humans when the insulin concentration is measured by a sensitive and specific ELISA method and submitted to appropriate deconvolution-based analysis.

Sampling directly from the portal circulation revealed a remarkable magnitude of insulin oscillations at this site. In the present study, the amplitude of insulin concentrations in the portal vein were ~100-1000 pmol/l in the basal state and increased to ~500-5000 pmol/l during hyperglycemia. These values in the human are comparable with those observed in the canine portal vein (5,6) and presumably reflect the insulin concentration wavefront to which the liver is exposed. Although the present study subjects were necessarily only those with portal hypertension requiring treatment, we selected subjects with normal or near normal liver function tests who were clinically well and had normal pre-study OGTT’s. Moreover, the range of insulin oscillations observed in the portal vein in these subjects is comparable with that previously observed in healthy dogs (5,6) and the portal vein blood flow, when available, was in the normal reported range for humans. Although some studies
have reported that pulsatile insulin delivery (to the peripheral circulation) enhances insulin sensitivity, none has yet recapitulated the magnitude of insulin oscillations to which we observe the liver is exposed in vivo during stimulated enhanced insulin secretion (21,22). The question remains, therefore, what the physiologic importance is, if any, of exposing the liver to such dramatic oscillations of insulin concentration. Since the magnitude of peripheral insulin pulses is decreased in patients with Type 2 diabetes (23), it is likely that the liver in these patients is exposed to even more attenuated oscillations in insulin pulse amplitude which may contribute to the hepatic insulin resistance characteristic of this disease (24).

The remarkable transhepatic attenuation of the insulin pulse signal from the portal circulation to the systemic circulation observed here is comparable to that observed previously in the dog (5). Although the attenuation may reflect up to ~4-fold dilution of portal venous blood by the systemic venous drainage, the insulin pulse amplitude is damped to a much greater extent than this (~10 fold). Presumably the latter reflects hepatic insulin clearance of insulin pulses. We previously noted that the hepatic clearance of portal vein insulin in the dog is related in an ascending monotonic (quadratic) manner to the pulse amplitude of the insulin concentration wavefront presented to the liver (25). Thus, we speculated that there is preferential hepatic clearance of the pulsatile component of insulin delivery. This notion is supported by our observation in the current study that there is a marked drop in the proportion of post-hepatic pulsatile insulin delivery (measured by arterial sampling) derived versus the pre-hepatic pulsatile insulin secretion (measured in the portal vein). In one prior study in which blood was sampled from the portal vein of patients with cirrhosis, comparable, large oscillations in insulin concentration were observed,
although no quantification of pulsatile insulin release or corresponding arterial sampling was available (26).

In summary, direct sampling from the portal vein in humans establishes an insulin interpulse interval of ~5 minutes, and thereby resolves the discrepant insulin pulse frequency previously reported in humans. A comparable insulin interpulse interval can also be detected by high-frequency peripheral sampling with the use of a highly sensitive insulin assay and appropriately validated pulse detection. Direct portal vein sampling also reveals that in humans the majority of insulin is secreted in insulin bursts, and that apparently preferential hepatic extraction of pulses leads to post-hepatic delivery of ~50% of insulin in discrete bursts. Extraction may be even higher in healthy subjects. Finally, we confirm that the liver in humans, like that of dogs, is exposed to dramatic insulin concentration oscillations that far exceed the magnitude evaluated to date in clinical studies of insulin action in vivo.

References


19. Veldhuis JD, Johnson ML, Faunt LM, et al. Assessing temporal coupling between two, or among three or more, neuroendocrine pulse trains: cross-correlation analysis,


Chapter 3

Acute effects of diazoxide on pulsatile insulin secretion in patients with Type 2 diabetes mellitus
Introduction

Type 2 diabetes mellitus is characterised by abnormal insulin secretion. The patterns of secretory defects include loss of first phase insulin secretion (1), increased irregularity of insulin release (2,3) and diminished insulin pulses (4).

Glucose-induced insulin release is partly achieved through the generation of ATP molecules following glucose oxidation and its subsequent interaction with the ATP sensitive potassium channels on the β cell membrane (5-7). This in turn leads to depolarisation of the β cell membrane, an influx of ionised calcium through the voltage gated calcium channels resulting in the elevation of cytoplasmic Ca$^{2+}$ concentration and exocytosis of the membrane bound (docked) insulin vesicles. Diazoxide, a potassium channel opener, inhibits insulin release by preventing the closure of ATP sensitive potassium channels in beta cells (8). Diazoxide has been shown to partially restore insulin secretion in patients with Type 2 diabetes when administered over one week period (9). It has also been shown to prevent the development of diabetes in Zucker Fatty rats (10) as well as rats previously subject to 90% partial pancreatectomy (11). The mechanism of this beneficial effect of diazoxide is not known. It has been hypothesised that, by “resting β cells”, islet insulin stores are preserved (11). Alternatively, the beneficial effects of diazoxide may be achieved by restoration of the **pattern** of insulin secretion. For example, by decreasing the intrinsic excitability of β cells, diazoxide may enhance the regularity of insulin release and/or the magnitude of discreet insulin pulses. This could have secondary beneficial effects by enhancing insulin sensitivity, and thus, decreasing insulin requirements.
In the present study we thus sought to test the following hypotheses. During exposure to diazoxide, insulin secretion in patients with Type 2 diabetes is characterised by (1) increased regularity, and (2) enhanced pulsatility of insulin secretion. To accomplish this, we examined the pattern of insulin secretion in patients with diet controlled Type 2 diabetes during a hyperglycemic clamp (thereby fixing the glucose stimulus for insulin secretion) on two occasions. On one occasion, following administration of an infusion of diazoxide (in view of its long half-life remained active during the period of study) and on the other occasion, in the absence of diazoxide.

Methods
Study subjects and design
Five patients with diet-controlled type 2 diabetes were studied in the Edinburgh University Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh. This study was approved by Lothian Ethics Committee and all volunteers provided written informed consent. Inclusion criteria for patients with type 2 diabetes were the fulfilment of WHO diagnostic criteria for either fasting plasma glucose above 7.8 mmol/l and/or plasma glucose above 11.1 mmol/l 2 hours after ingestion of 75 gram carbohydrate, onset of diabetes after 35 years of age and absence of diabetic ketoacidosis. Exclusion criteria were pregnancy, abnormal liver or renal function tests, history of pancreatitis, ischaemic heart disease, cerebrovascular disease, hypotension, uncontrolled hypertension, epilepsy, anaemia and treatment with sulphonylurea or insulin. The characteristics of all the study subjects are listed in Table 1.
TABLE 1

Characteristics of study subjects (mean ± SE)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>55.8 ± 3.5</td>
</tr>
<tr>
<td>Duration of diabetes (yrs)</td>
<td>2.8 ± 0.7</td>
</tr>
<tr>
<td>Body mass index (m/kg$^2$)</td>
<td>31.0 ± 1.6</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.0 ± 0.4</td>
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</tbody>
</table>
Study protocol

All patients were admitted to the Edinburgh University Wellcome Trust Clinical Research Facility at 8 am on the study day after an overnight fast. All anti-hypertensive medications were omitted on the morning of the study. They were studied twice; on one occasion after a diazoxide infusion and on the other occasion after a saline infusion. The studies were carried out 2-3 weeks apart. On each occasion, study subjects were placed in the supine position on a bed and 2 intravenous infusion catheters were inserted. They were placed in an antecubital vein (one in each arm) for the subsequent infusion of glucose and saline with or without diazoxide respectively. A third intravenous catheter was placed in a dorsal hand vein (the hand being warmed by an electric blanket to 40 °C) for the purpose of sampling arterialised blood. At protocol time 0 (approximately 9:00 am) blood was obtained at 1-minute intervals for 40 minutes (t=0-40 min) for subsequent measurement of the plasma insulin concentration. Additional samples were obtained at 20-minute intervals for measurement of the plasma C-peptide concentration as well as 5-minute intervals for measurement of the plasma glucose concentration throughout the study. At t=40 min, a glucose infusion was commenced to initiate a hyperglycaemic glucose clamp with a target plasma glucose concentration of 9-10 mmol/l. This was achieved by a variable rate infusion of exogenous glucose (50% dextrose) varied in response to the results of the five-minutely plasma glucose concentrations. The hyperglycaemic clamp was maintained throughout the remainder of the study (t=40-410 min). During protocol period t=80-110 min, diazoxide was infused at 2 mg/kg/min. 4-5 hours after the diazoxide infusion, a second intensive 40 minute intensive (minutely) sampling protocol was undertaken (again to obtain plasma for subsequent insulin assay).
Throughout the study, the blood pressure and pulse rate were monitored at 15-30 min intervals by an automated BP monitor (Dynamac). Blood samples for subsequent insulin and C-peptide assay were collected into chilled EDTA tubes, centrifuged and the plasma stored at -20 °C for subsequent assay. Following this second intensive sampling period the study was completed (at approximately 4 p.m.) and patients ingested a meal prior to leaving the Edinburgh University Wellcome Trust Clinical Research Facility.

Assays

Glucose. Plasma glucose concentrations were measured by the glucose oxidase method by a Beckman glucose analyser (Beckman, Palo Alto, CA).

Insulin. Serum insulin concentrations were measured in duplicate by a two-site immunospecific insulin ELISA as previously described. In brief, the assay uses two monoclonal murine antibodies (Novo Nordisk, Bagsvaerd, Denmark) specific for insulin. The detection range of this insulin ELISA was 5-600 pmol/l. At medium (150 pmol/l), medium-high (200 pmol/l) and high (350 pmol/l) plasma insulin concentrations, the interassay coefficient of variation were 3.7%, 4.0% and 4.5% respectively and the corresponding intra-assay variations were 2.3%, 2.1% and 2.0% respectively. There was no cross-reactivity with proinsulin and split 32,33 and des 31,32 proinsulins, respectively.

C-peptide. Plasma C-peptide concentrations were performed using a commercially available kit (K6218; DAKO, Cambridgeshire, UK). The assay is a two-site ELISA based on two monoclonal antibodies. Each sample was assayed in duplicate; intra- and inter-assay variation coefficients were 2.2% and 3.3% respectively.
Data analysis

Detection and quantification of pulsatile insulin secretion by deconvolution.

The plasma insulin concentration time series were analysed by deconvolution to detect and quantify insulin secretory bursts. Deconvolution of venous insulin concentration data was performed with a multiparameter technique (12) that requires the following assumptions. Plasma insulin concentrations measured in samples collected at 1-min intervals were assumed to result from five determinable and correlated parameters: 1) a finite number of discrete insulin secretory bursts occurring at either regular or randomly dispersed times and having a) individual amplitudes (maximal rate of secretion attained within a burst) and mass (integral of the calculated secretory event) and b) a common half-duration (duration of an algebraically Gaussian secretory pulse at half-maximal amplitude) that are superimposed on c) a basal time-invariant insulin secretory rate; and 2) a bi-exponential insulin disappearance model in the systemic circulation, consisting of earlier directly estimated half-lives of 2.8 and 5.0 min and a fraction slow component of 0.28 in healthy fasting humans (13). All secretory rates were expressed as mass units of insulin (picomoles) released per unit distribution volume (litres) per unit time (minutes), where the volume of distribution was 0.206 l/kg. Total insulin secretion was calculated by deconvolution of C-peptide concentrations (14).

Total body insulin clearance was estimated from the calculated pre-hepatic insulin secretion rates (from deconvolution of C-peptide concentrations) and from peripheral plasma insulin concentrations.

\[ C \text{ (L/min)} = \frac{S \text{ (pmol/min)}}{I \text{ (pmol/L)}} \]
where C is the net insulin clearance rate, S is calculated insulin secretion rate and I is the peripheral plasma insulin concentration.

**Quantification of irregularity.**

The orderliness of insulin secretion was assessed by the application of approximate entropy, which is a model-independent and scale-invariant statistic (15). Approximate entropy assigns a single non-negative real number to a time series in which larger absolute values correspond to greater apparent process randomness, and smaller values correspond to more instances of recognizable patterns or consistent features in the data. Briefly, approximate entropy measures the logarithmic likelihood that runs of patterns that are close (within r) for m contiguous observations remain close (within the same tolerance width r) on next incremental comparisons. For this study, we calculated approximate entropy values for all data sets, with m=1 and r=20% of the SD of the individual subject time series. We applied approximate entropy with these input parameters to the first-differenced serum insulin concentration time series to minimize the effects of short-term nonstationaries in the data.

**Statistical analysis**

Data are presented as the mean ± SEM. Statistical comparison between groups was analysed either by a paired Student’s two-tailed t-test (comparisons between diazoxide versus saline study at fixed times) or one factor analysis of variance (comparisons over time in one or other group). A probability of less than 5% due to occurrence of chance alone (p<0.05) was taken as evidence of statistical significance.
Results

Experimental conditions

Blood pressure and plasma glucose concentration.

The mean plasma glucose concentration prior to the clamp (during the basal intensive sampling period) was comparable on the two study occasions (saline vs diazoxide, 8.3±0.9 vs 8.3±1.1 mmol/l). During the hyperglycemic clamp the plasma glucose concentration was successfully maintained in the target range and was again comparable on the two study days (saline vs diazoxide, 9.4 ± 0.8 vs 9.7 ± 0.9 mmol/l) (figure 1).

There was no difference in the mean systolic blood pressure during the basal study period (t=0-40 min) (saline vs diazoxide, 129.7±3.1 vs 135.6±1.7 mm Hg, p=0.14 ANOVA). The mean systolic blood pressure decreased during and following diazoxide infusion (saline vs diazoxide, 131.5±1 vs 121.4±1.2 mm Hg, p<0.001 ANOVA), an effect which persisted until the end of the study (figure 2).

Diazoxide effects on insulin and C-peptide concentrations.

The mean basal plasma C-peptide concentrations prior to the diazoxide or saline infusions was similar (diazoxide vs saline, 1.17±0.12 vs 1.16±0.13 nmol/l, p=0.65). During the hyperglycemic clamp there was an expected increase in the C-peptide concentration on the saline study day (from 1.16±0.13 to 1.60±0.3 nmol/l, basal versus final hour of clamp, p<0.01). However, during the clamp, following the initiation of the diazoxide infusion until the end of the study, the C-peptide concentrations were lower compared to the corresponding values obtained following a saline infusion; (first hour after diazoxide;
Fig 1. Plasma glucose concentration throughout study.
Fig 2. Mean systolic blood pressure throughout study.
diazoxide vs saline, 1.09±0.18 vs 1.47±0.24 nmol/l, p<0.01; *five hours after diazoxide,*
diazoxide vs saline, 1.4±0.3 vs 1.6±0.3 nmol/l, p<0.05). This relatively steady state
suppression of insulin secretion for the five hours after diazoxide indicated by the decreased
C-peptide concentration is consistent with the prolonged biological half life of diazoxide
(28 hours) (figure 3).

As expected the mean plasma insulin concentration was comparable in the basal state prior
to the clamp on both the diazoxide and saline visits (diazoxide vs saline, 90.7±15.7 vs
77.4±13.3 pmol/l, p=0.1). However, in contrast to the C-peptide concentrations, during the
final hour of the clamp the mean plasma insulin concentration after the diazoxide and saline
infusions were comparable (diazoxide vs saline, 140±60 vs 134.7±49 pmol/l, p=0.6) (figure
4). As the C-peptide and insulin concentrations were stable at this time these data imply that
diazoxide caused a decrease in insulin secretion but no change in insulin concentration in
the systemic circulation.

*Diazoxide effects on pulsatile insulin secretion*

Inspection of the individual insulin concentration profiles in each patient confirmed the
presence of oscillations in insulin concentration with a pulse interval of approximately 6
minutes. When these insulin concentration profiles were subject to analysis with a
deconvolution program previously validated for this purpose, pulses were indeed detected in
each data set analyzed.
Fig 3. C-peptide concentration throughout study.
Fig 4. Mean plasma insulin concentration during final hour of hyperglycaemic clamp.
In the basal fasting state, there was no difference in the calculated mean insulin secretory rate (diazoxide vs saline, 3.3±0.2 vs 3.3±0.3 pmol/kg/min, p=0.7), pulse mass (diazoxide vs saline, 48.3±10.3 vs 30.8±8.6 pmol, p=0.2), pulse interval (5.5±0.2 vs 5.4±0.2 mins, p=0.8) and pulse amplitude (diazoxide vs saline, 13.9±2.9 vs 9.4±1.6, pmol/l, p=0.14) consistent with the comparable C-peptide concentrations under these conditions. However, following diazoxide infusion, during the clamped period of the second intensive sampling period, the insulin secretion rate was decreased (diazoxide vs saline, 4.2 ±0.7 vs 4.8±0.6 pmol/kg/min, p<0.05) consistent with the observed reduction in C-peptide level. However, the pulse mass (diazoxide vs saline, 47.0±17.7 vs 43.7±9 pmol, p=0.7), pulse interval (diazoxide vs saline, 5.9±0.5 vs 5.0±0.3 mins, p=0.05) and pulse amplitude (diazoxide vs saline, 13.4±6.4 vs 16.1±3.5 pmol/l, p=0.4) were not different from the corresponding data obtained after the saline infusion. Interestingly, insulin clearance was significantly decreased following diazoxide infusion (diazoxide vs saline, 3.5±0.7 vs 3.9±0.9 L/min, p<0.01).

**Effects of diazoxide on the orderliness of insulin concentration profiles.**

The orderliness of insulin concentrations were not changed after diazoxide (diazoxide vs saline, 1.1±0.05 vs 1.1±0.03, p=0.9).

**Discussion**

The present study was undertaken in order to test the hypothesis that in patients with Type 2 diabetes, diazoxide results in enhanced pulsatile and/or orderliness of insulin secretion. We conclude that, while diazoxide did cause an expected decrease in the rate of pre-hepatic insulin secretion, unexpectedly under these conditions neither the rate of post hepatic
insulin delivery or post hepatic insulin pulse mass (or amplitude) was altered. No changes in orderliness of insulin secretion were seen under these conditions.

The apparent disparity between the decreased pre-hepatic insulin secretion rate (C-peptide) but comparable post-hepatic insulin secretion rates during exposure to diazoxide may have several explanations. One possible explanation is that the clearance rate of C-peptide and/or insulin may theoretically be altered by diazoxide. However, there is no data to support this. An alternative explanation is that the hepatic clearance rate of insulin is decreased in the presence of diazoxide. We have previously reported unchanged insulin concentrations in the systemic circulation during partial suppression of insulin secretion (measured directly in the portal circulation in a canine model) during a low dose somatostatin infusion (16). In those studies, as we had direct access to the portal circulation, we were able to reveal that there was a strong relationship between the pulse amplitude of the portal vein insulin concentration and hepatic insulin clearance rate. It has long been recognised that the concentration of insulin in the systemic circulation depends on both the rate of insulin secretion and the hepatic insulin clearance rate. Since virtually all insulin is secreted in discrete insulin bursts (13,17), we previously proposed that the decreased rate of hepatic insulin clearance of endogenously secreted insulin may serve as an adaptive mechanism to sustain the concentration of insulin in the systemic circulation under conditions of diminishing secretion rates (for example during evolving Type 1 or Type 2 diabetes mellitus) (16). More recently we have identified additional support for this concept in a porcine model of partial β cell loss achieved with alloxan (18). In these animals we again observe that insulin concentrations in the systemic circulation are preserved in the setting of decreased insulin secretion rates and that this disparity is due to decreased hepatic insulin
clearance rates of endogenously secreted insulin. In that porcine model with the benefit of portal vein catheterisation we again observed a direct relationship between the insulin pulse amplitude and the rate of clearance of endogenously secreted insulin, namely, that smaller pulses were associated with reduced clearance. Therefore, it is plausible that the discrepancy between the C-peptide and insulin concentration data observed in this study is due to diazoxide-induced decrease in pre-hepatic insulin secretion leading to smaller pulse amplitude in the portal vein insulin concentration resulting in a compensatory decrease in hepatic insulin clearance of endogenously secreted insulin. Such a circumstance might be deemed beneficial under conditions of impaired β cell function as comparable insulin concentrations are achieved in the systemic insulin concentrations despite a decreased rate of insulin secretion.

It is known that insulin secretion is irregular and chaotic in patients with Type 2 diabetes (2). The statistic approximate entropy has been used to document increased disorderliness of insulin concentration profiles (19). We hypothesized that diazoxide might decrease the disorderliness of insulin concentration profiles by reducing the excitability of β cell membranes by retaining the potassium channels open (and so increasing resting membrane potential). However under the conditions of the present study there was no effect of diazoxide on orderliness of insulin concentrations.

It is of related interest that diazoxide treatment had no effect on the insulin pulse interval. Therefore, the rhythmic activity of pancreatic pacemaker is not influenced by diazoxide. The pacemaker responsible for the generation of insulin pulses remains controversial. Various hypotheses have been proposed for the origin of pancreatic pacemaker, namely,
oscillations in glycolysis (metabolic) (20), cytoplasmic Ca\textsuperscript{2+} (21) concentration or intra-pancreatic ganglia (22). Since oscillations in insulin concentration were observed despite under the inhibitory influence of diazoxide, the metabolic origin as the pancreatic pacemaker is more likely although the contribution of intra-pancreatic ganglia cannot be ruled out. This is consistent with in vitro studies demonstrating the occurrence of oscillatory insulin secretion from pancreatic islets exposed to diazoxide where the membrane potential has been ‘clamped’ by diazoxide (23).

In summary, we report that peripheral insulin concentrations did not decrease despite reduction in pre-hepatic insulin secretion following diazoxide infusion. This could be ascribed to the reduction in hepatic insulin clearance following the reduction in insulin pulse amplitude in the portal circulation. Finally, inter-pulse interval remained unchanged after diazoxide implying that the pancreatic pacemaker activity is a stable phenomenon.

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Chapter 4

Pulsatile insulin secretion by human pancreatic islets
Introduction

Insulin is secreted in a pulsatile manner (5,10,11,14,22). These pulses can be detected *in vivo* as well as *in vitro* in perifused islets of Langerhans (2,3,5,6,8,9,15). Reliable detection and quantification of pulsatile hormone secretion requires statistical methods that have been validated for the conditions in which they will be used. We have previously validated the use of a deconvolution technique developed by Johnson and Veldhuis (29) for detection and quantification of pulsatile insulin secretion *in vivo* (22). In the present study, we sought to extend this approach for the quantification of pulsatile insulin secretion by perifused human islets of Langerhans. Perfusion studies have the advantage that they allow analysis of the mechanisms that regulate insulin secretion in the absence of the complexities of feedback control (for example by the changes in plasma glucose concentration). They also allow mechanistic studies into the factors that generate pulsatile insulin secretion (pacemaker activity) as well as coordinate this signal between islets (coordination).

In the present study, we appraised the sensitivity and specificity of two statistically independent methods for pulse detection, Cluster analysis (31) and deconvolution analysis (7). Optimized pulse detection was then applied to examine pulsatile insulin secretion in response to relevant stimuli (glucose and potassium) to explore the mechanism(s) driving enhanced insulin release.

Methods and materials

Protocol 1: Sensitivity and specificity of pulse detection

The purpose of this protocol was to establish the criteria for valid insulin pulse detection from perifused human islets. Fluctuations in insulin concentration in perfusate collected
from perifused islet include contributions from assay variance, experimental variability, and genuine oscillations in the insulin secretion rate. Therefore the influence of each of these components on two published methods for detection of pulsatile insulin secretion, Cluster analysis and deconvolution, were examined in protocol 1a, 1b and 1c. The objective was to minimize detection of pulses from system or assay noise (false positive detection), while maximizing true-positive detection of insulin pulses. To this end, 3 experimental protocols were performed in which the variance of each component outlined above was examined within the insulin concentration range (5-100 pmol/l), which corresponds to those observed in the perfusate from human islets.

*Protocol 1a: Specificity; assay variance.*

Human insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was diluted to five concentrations (5, 20, 30, 60 and 100 pmol/l) in Krebs' ringer bicarbonate buffer (115 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, 5 mmol/L NaOH) supplemented with 0.2% human serum albumin. Forty aliquots were collected from each of the five insulin concentrations and stored at −20 °C until assayed in duplicate.

*Protocol 1b: Specificity; system variance.*

To determine variance in insulin concentration levels attributable to the perifusion system the 5 insulin dilutions prepared in 1a were pumped through the perfusion system (duplicate experiments) in an identical manner to that in which islets were perfused. Specifically, the perfusate was gassed with O₂/CO₂ (95:5) and maintained at pH 7.4 before being pumped through the system at constant flow rate of 0.3 ml/min for 42 minutes. The effluent was collected at 1-minute intervals and then stored at −20 °C prior to assay.
Protocol 1c: Sensitivity of true pulse detection.

We simulated oscillations of insulin concentration (in a range comparable to those observed from human islets) in the effluent from the perifusion system by superimposing four amplitudes of insulin oscillation (7.5, 12.5, 22.5 and 50 pmol/l) onto each of the 5 constant insulin infusions (total of 20 perfusions) described in 1b. In each experiment 7 pulses were infused over a period of 42 minutes. Pulses were generated in a square wave form for 0.5 minute superimposed on the constant insulin infusion. After the 0.5 minute pulse there was a 5.5 minute period with the constant basal insulin infusion only, so achieving an interpulse interval of 6 minutes to mimic that observed in human islets. Perfusate effluent was collected at 1-minute intervals for 42 minutes and stored at −20 °C until assay in duplicate for insulin.

Apparatus.

The perifusion system consisted of two separate pumps, Endotronic Automated Pumping System (APS) and ACUSYST-S pumps (Celllex Biosciences, Inc, Minneapolis), linked together via an integration chamber. The Endotronics APS is a fluid handling system providing a wide range of capabilities ranging from fixed pump rates to sophisticated signal control including delivery of a pulsatile infusion pattern. It consists of a control panel and 3 peristaltic pumps (medium, substrate and waste). It has the ability to control the 3 pumps independently (i.e. to produce a constant rate of infusion) or use a coordinated control scheme (i.e. to produce pulsatile pattern of delivery) and this capability is achieved through ACUSOFT-APS software package in an on line personal computer. The ACUSYST-S perifusion system consists of a multi-channel peristaltic pump, tubing sets, a heat exchanger and perifusion chambers. The peristaltic pump draws the perfusate from the integration
chamber and delivers it through the tubing sets. The perfusate is carried through the heat exchanger where it is warmed. From the heat exchanger, the perfusate is delivered to the islet chambers. Output from the islet chambers is collected in a fraction collector for analysis. The infusion rates in the Endotronics APS and ACUSYST-S pumps were coordinated via ACUSOFT-APS software to achieve a flow rate of 0.3 ml/min and generate the pulsatile pattern of insulin delivery. The medium and the substance pumps delivered perfusate of different insulin concentrations to the integration chamber, where it was mixed prior to delivery to the perifusion system to achieve the desired pulse waveform. Effluent was collected at one-minute intervals.

Protocol 2: Recovery of infused insulin pulses

The purpose of protocol 2 was to define the dynamics of islet secretion, based on the observed fluctuations of insulin concentration in the perifusate. The insulin output from the perifusion system is a function of both the actual secretion dynamics of the islet in the islet chamber and the damping of this signal in the subsequent drainage system as well as any losses of insulin via adsorption to the apparatus. Accordingly, we first delivered insulin pulses directly into the islet chamber in a profile that mimicked the secretory behavior of islet beta cells predicted by electrophysiology studies (2,12,26). We then used the resulting profile of insulin concentration data observed in the perifusion effluent to derive the parameters required to recover the known insulin secretion profile (delivered into the islet chamber) by use of deconvolution.
Apparatus.

The ACUSYST-S pump was used to maintain the perfusate at a flow rate of 0.3 ml/min. A high precision programmable infusion pump (Harvard pumps, Cambridge, MA) was used to deliver insulin (1000 pmol/l) at the rate of 0.1 ml/min for 2 min every 6 min for insulin pulse generation.

The Harvard pump was connected to the perifusion chamber directly via an infusion line, so that the insulin was pumped directly into the chamber. During the time the Harvard pump was active, the flow rate pumped by ACUSYST-S pump was reduced to 0.2 ml/min in order to preserve an overall flow rate of 0.3 ml/min.

Protocol 3: Pulsatile insulin release by human islets

In order to examine the pattern of insulin secretion from human islets in the above perifusion system, we studied single human islets exposed to basal (5.5 mmol/l) and stimulated (8, 16 and 24 mmol/l) glucose concentrations (n=4). Single islets were also studied at basal (5 mmol/l) and high (30 mmol/l) potassium concentrations at a constant glucose concentration of 5 mmol/l. Since many islet perfusion protocols involve groups of islets we also examined groups of 5-20 human islets per chamber perifused at 20 mmol/l glucose (n=20).

Islet isolation.

Human islets were isolated from pancreas tissue retrieved from 4 heart-beating organ donors by the department of surgery at the University of Leicester, U.K. The donor did not have a history of diabetes. After isolation, islets were cultured in RPMI 1640 medium
containing 5.5 mmol/l glucose, 2 mmol/L glutamine, 10% fetal calf serum, 100 U/ml benzylpenicillin and 0.1 mg/ml streptomycin. The islets were cultured in Leicester for 2 days before being sent to Edinburgh. Islets were then transferred to fresh RPMI 1640 medium (same composition as above) and cultured for 2 days before experiments were performed.

**Islet perifusion.**

Single human islets were removed from the static incubation media, and suspended in Bio-Gel P-2 beads (Bio-Rad, Hercules, CA) and placed in perifusion chambers. The perifusion system consisted of a multi-channel peristaltic pump that delivered perfusate through 6 parallel tubing sets via a heat exchanger and six perifusion chambers a rate of 0.3 ml/min after warming to 37 °C and gassing with 95% O₂. The perfusate was delivered to the perifusion chambers containing the human islets. The perifusion protocol consisted of two study arms; the islets were exposed to (a) basal glucose (4 mmol/l) concentration for 40 min and thereafter to 8, 16 or 24 mmol/l for another 40 min; (b) basal (5 mmol/l) potassium concentration for 40 min and thereafter, to high (30 mmol/l) potassium concentration for another 40 min. When the islets were exposed to 30 mmol/l potassium, the NaCl concentration was reduced to 96 mmol/l to keep the osmolarity constant in the Kreb’s Ringer buffer. In each protocol, an equilibrium period of 30 min was allowed before study. Effluent perifusate was collected at 1-minute interval and stored at -20 °C and subsequently analyzed for insulin concentration.
Assays

Insulin. Insulin concentrations were measured in duplicate by two-site immunospecific insulin ELISA as previously described (17). The assay uses two monoclonal murine antibodies (Novo Nordisk, Bagsvaerd, Denmark) specific for insulin. There is no cross-reactivity with proinsulin and split 32,33 and 31,32 proinsulins. Sensitivity of the assay is 5 pmol/l and the detection range is 5-2000 pmol/l. The intra-assay and inter-assay CVs ranged from 2.0-2.3% and 3.7-4.5% respectively. In all the present studies all measured insulin concentrations were within this operating range of the insulin assay.

Calculations and statistical analysis

Deconvolution analysis calculates underlying insulin secretion rates and the position, duration, mass and amplitude of insulin secretory bursts as well as the basal or non-pulsatile insulin secretion rate based on known or assumed half-life parameters (13,29,30). A column of apparent half-life was estimated by monoexponential fitting of the observed insulin concentrations in serial one-minute samples following abrupt cessation of a continuous insulin infusion in two experiments, thereby correcting for secondary skewness imposed on the perifusion insulin waveform due to non-specific time-delay of column flow-through (24).

Cluster analysis, in contrast, determines statistically significant up- or down strokes in the serial insulin concentrations, providing information only about the frequency and amplitude of the oscillations in the data series without any assumptions or knowledge of the insulin half-life in the system of study or underlying secretory burst waveform. The t-statistics used for evaluating significant upstrokes and down strokes in the insulin time series were
taken as 2.0, based on prior simulation studies. The corresponding estimated cluster sizes of 2 and 2 in the nadirs and peaks were defined using signal-free insulin profiles (below).

**Results**

**Protocol 1: Sensitivity and specificity for pulse detection**

(a) Protocol 1a: Detection of false-positive pulses due to assay variance.

Representative insulin concentration profiles obtained in protocol 1a, 1b and 1c are shown in figure 1. Despite the low coefficient of variation of the current insulin assay, fluctuations in the insulin concentration obtained by multiple replicates from the same sample of buffer can give the appearance of pulses when plotted sequentially as in figure 1 (top panel). When using Cluster analysis to examine these data, the criteria required to avoid false detection of insulin oscillations are shown in table 1a. Use of a t-score of 2 or greater resulted in detection of less than 2 false positives for detection insulin oscillations irrespective of the cluster size selected to define a pulse. As shown below a cluster size of 2x2 was required for reliable detection of true pulses. Thereby, we observe that a mean of 0.8 false positives (never more than 1) was detected in the 6 assay profiles examined. When the same insulin concentration profiles were subject to pulse detection using deconvolution (using a single half life of 0.63 min and a volume of distribution of 0.33 ml as established in protocol 2), a mean of 1.3 false-positives was detected for the 40 samples assayed at each concentration.
Fig 1.

Panel A: 40 assay replicates from the same buffer sample plotted in sequence of assay; Panel B: insulin concentrations (mean of duplicates) obtained sequentially from the perfusion apparatus after perfusion of the same buffer shown in A at a constant rate through the perfusion apparatus; Panel C: 40 insulin concentrations (mean of duplicates) obtained in sequence from the perfusion apparatus during a pulsatile insulin infusion. Site of pulse identified by deconvolution circle, cluster star.
During a pulsatile insulin infusion, the most acceptable assessment of pulsatile insulin detection was obtained with a cluster size of 2 x 2 and a t-score of 2.

Collected at one-minute intervals for subsequent assay in duplicate, Protocol 1c shows mean number of true pulses identified (out of 7) detected when the same buffer preparations were pumped through the perfusion system at a constant rate and 40 consecutive aliquots were collected from 5 buffer preparations containing insulin at 5, 20, 30, 60, and 100 pmol/l. Protocol 1b shows the mean number of false positive pulses from 5 buffer preparations containing insulin at 5, 20, 30, 60, and 100 pmol/l. Protocol 1a shows t-scores from 1 to 4. Protocol 1a shows t-scores from 1 to 4.

The most acceptable assessment of pulsatile insulin detection was obtained with a cluster size of 2 x 2 and a t-score of 2.

### Table 1: Cluster Analysis for Protocol One

<table>
<thead>
<tr>
<th>Cluster Size</th>
<th>Protocol 1a</th>
<th>Protocol 1b</th>
<th>Protocol 1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 1</td>
<td>0.11</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>1 x 2</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>2 x 2</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>3 x 3</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>4 x 4</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
</tr>
</tbody>
</table>

**Legend:** Number of pulses detected by Cluster analysis of insulin concentration profiles from Protocol 1 using cluster sizes from 1 x 1 to 8 x 8 and t-scores from 1 to 4.
(b) Protocol 1b: Detection of spurious pulses due to variance in insulin assay and variance due to the perifusion system.

A representative perifusion insulin concentration profile during constant insulin delivery is shown in figure 1 (middle panel). The results of Cluster analysis applied to these data are summarized in table 1b. A t-score of 3 or greater irrespective of cluster size detected less than one pulse with each experiment. A t-score of 1 provided unacceptable rates of false positives at cluster sizes of 4 by 4 or less. A t-score of 2 revealed false positives pulses at a rate of less than 2 per 42 minute data series with cluster sizes of 4x4 or greater, and (2-3 false positive errors) at cluster sizes of 2x2 and 3x3. When the same data were subjected to deconvolution analysis, the rate of false positives averaged 2.5 pulses per 42-minute profile.

(c) Protocol 1c: Sensitivity of true-positive pulse detection.

A representative insulin concentration profile obtained during pulsatile insulin perifusion (seven pulses) is shown in figure 1 (lower panel). Cluster analysis yielded the data summarized in table 1c. A t-score of 3 or greater resulted in a pulse detection rate of less than 50% in most circumstances. A t-score of 2 allowed detection of 64% of pulses at a cluster size of 2x2. Although a t-score of 1 and cluster size of 1x1 detected 99% of pulses, these criteria led to 6 false-positive pulses over the same period during a constant infusion. Therefore, the most appropriate Cluster criterion for detection of insulin pulses from human islets in this perifusion system is a t-score of 2 and cluster size of 2x2. Use of these criteria provided detection of 64% of true pulses (mean detection rate of all amplitudes infused) during a pulsatile infusion, while yielding 2.7 false positives on average during a constant insulin infusion. The design of protocol 1c allowed for examination of the impact of the increment in pulse amplitude above baseline on pulse detection. This analysis under
optimized Cluster analysis (t=2 and cluster size of 2x2) gave the results shown in figure 2. When the mean amplitude of the pulse was 40% or greater than baseline, Cluster analysis detected ~80% of pulses. However, when the amplitude of the pulse was only 10-20% above baseline, Cluster analysis detected ~50% of pulses.

When deconvolution was used to examine insulin concentration profiles obtained during the pulsatile insulin infusions, 94% of true pulses were detected (mean detection rate of all amplitudes infused). The impact of the amplitude of infused oscillation(s) on pulse detection is shown in figure 2. Deconvolution detected 85-100% of true pulses when the amplitude of the insulin pulse above baseline was 20% or more (either 6 or 7 pulses detected out of 7 true pulses). In conclusion, deconvolution was more sensitive (6% false negatives versus 34% false negatives with Cluster analysis) and comparably specific (2.5 versus 2.7 false positive per 42 insulin samples during constant infusion) compared to Cluster analysis.

**Protocol 2: Recovery of infused insulin pulses**

In this protocol, we tested recovery of insulin secretion kinetics during known pulsatile insulin infusions given directly into the islet chamber so as to reproduce so closely as possible the presumed pattern of insulin secretion by human islets. Since deconvolution but not Cluster analysis measures secretion we employed deconvolution only in protocol 2. The measured volume of distribution of insulin delivered in this manner to the perifusion system was 0.33 ml. The decay curve for insulin concentration following cessation of an insulin pulse fitted conformed to monoexponential decay supporting a single compartment model with a calculated half-life of 0.63 minutes. Figure 3 allows direct comparison of the
Sensitivity of pulse detection by cluster analysis (open bars) and deconvolution (closed hatched bars) for detecting infused pulses as a function of the percent increment in amplitude above baseline. 7 pulses (broken line) were actually infused.
known insulin delivery rates (middle panels), the insulin concentration profile that resulted from this (top panels) and the calculated insulin secretion rates by use of deconvolution of these concentration data (lower panels). Deconvolution closely approximated insulin delivery into the islet chambers. In fact given the inevitable damping of the delivery into a fluid space, the deconvolution profile is likely more representative of the minute-averaged appearance of insulin in this chamber than the pumped infusion rate (i.e. Guassian versus truncated triangles). Furthermore, deconvolution of the insulin concentration profiles yielded excellent recovery of the known insulin delivery rate (92.4±4.9%) as well as the known insulin infused pulse mass (94.5±1.6%). The deconvolution program ascertained that 93±2.5% of insulin delivery was derived from pulses, whereas in this protocol 100% of insulin was delivered in pulses.

Protocol 3: Pulsatile insulin release by human islets

Pulse detection by deconvolution versus Cluster analysis provided a comparable measure of pulse interval (6.7±0.5 vs 8.0±1.5 min, p= 0.12; deconvolution vs cluster), when multiple islets were perfused irrespective of the mean insulin concentration in the islet effluent (mean insulin concentration 26.1±3.9; range 10.4 to 48.8 pmol/l). Although there is no known pulse interval with real islets (in contrast to the pseudo islet studies in protocol 2), the likely pulse interval from electrophysiological studies is ~6 min (7,12,26), and agrees well with these data. In contrast when single human islets were perifused, the pulse interval with deconvolution remained comparable to that seen in multiple islet perfusions, whereas Cluster analysis detected fewer pulses and consequently a longer pulse interval (6.9±1.3 vs 11.9±1.0 min, p=<0.01, deconvolution versus cluster). Deconvolution provided a consistent
The insulin concentration profile (top panels), insulin secretion profile infused to mimic a pseudo-islet (middle panel), and the deconvolved insulin secretion rates (bottom panels) in two studies described in Protocol 2.

Fig. 3.
value for the pulse interval throughout insulin concentration range observed in the effluent from single islets (mean 12.2±1.0, range 5.4-22.3 pmol/l). We therefore used only deconvolution to examine the effects of increased glucose and potassium concentrations on pulsatile insulin release by single islets.

Recovery of insulin secretion from perifused human islets using deconvolution of the insulin concentration profile was 102±1.4% for single islets, and 104.8±1.4% for multiple islets (5-10) (figure 4). The insulin concentration profile in single human islets examined in the present system invariably revealed distinct oscillations (figure 5). After the perfusate glucose concentration was increased, the insulin secretion rate per islet increased (2.8±0.2 to 4.9±0.6 fmol/min, p<0.05) and this was achieved by an increase in pulse mass (6.5±2.7 to 19.5±7 fmol, p<0.05) with no change in pulse frequency (8.1±0.9 to 6.3±0.9 min, p=0.1). Increased perfusate potassium concentration from 5 to 30 mmol/l also stimulated increased insulin release (3.9±0.6 to 5.7±0.7 fmol/min, P<0.05), which was analogously achieved through the specific mechanism of increased pulse mass (6.2±1.4 to 14.5±2.4 fmol, p<0.05) with no change in pulse frequency (8.1±1.5 vs 8.0±1.7, p=0.9) (figure 6). Based on single human islets, the calculated proportion of insulin secreted in distinct pulses versus in a basal (non-pulsatile) insulin release was 25.6±4.7 and 37.2±8.8% (control versus stimulated with potassium), and 23.3±9.3 vs 56.2±7.2% (basal versus stimulated with glucose). The mean calculated proportion of insulin secreted in discrete bursts with multiple islet perifusions was 32.2±3.9%.
Fig 4.

The relationship between the insulin mass calculated by the product of the insulin concentration and the buffer flow rate (X axes) and the insulin mass obtained by deconvolution (Y axes). Relationship shown when multiple islets perfused in each chamber (top panel) and single islets per chamber (bottom panel).
Fig 5

The insulin concentration profiles (top panels) and the deconvolved insulin secretion rates (lower panels) in representative single human islets during basal (t=0-40 min) and stimulated (t=40-80 min), glucose (4 to 8 mmol/L) and KCl (5 to 30 mmol/L). The insulin pulse mass increased in response to stimulation with high glucose or potassium but there was no change in pulse frequency.
Fig 6.

The impact of a high potassium concentration on pulsatile secretion by isolated perfused islets. Pulse mass (left panel), pulse interval (right panel). Basal potassium 5 mmol/L, stimulated potassium 30 mmol/L. The insulin pulse mass increased in response to stimulation with high glucose or potassium but there was no change in pulse frequency.
Discussion

*In vivo,* most insulin is secreted in discrete secretory bursts (22). Regulation of insulin secretion is achieved physiologically through the mechanism of modulation of the amplitude of insulin pulsatility (2,17,23,24,25). Abnormal pulsatile insulin secretion is an early defect in patients developing Type 1 or Type 2 diabetes (4,17). Accordingly, an understanding of the mechanisms underlying the generation, co-ordination and regulation of insulin secretory bursts is potentially of importance. Estimation of pulsatile insulin secretion *in vivo* in humans and large animals presents particular experimental and analytical challenges, including the delivery of insulin into the portal vein and liver, wherein it undergoes extraction prior to delivery to the usual sampling site, the systemic circulation (16,18,21). Estimation of pulsatile insulin secretion in rodents is complicated by the limitations of blood volume. To overcome these problems pulsatile insulin secretion has frequently been examined in isolated perfused islets (2,3,5,6,9,15). However the quantification of pulsatile insulin release in isolated islets is not without technical challenges. The quantity of insulin released by isolated perfused islets is very small and so such studies are susceptible to the difficulties of distinguishing variability of concentration data due to noise in the assay system from small fluctuations due to episodic secretion at or near the limits of the capacity of assay resolution. Furthermore any perfusion system is vulnerable to experimentally uncontrolled fluctuations in output consistency associated with mechanical facets of the apparatus.

In the present study, we established the conditions in which pulsatile insulin secretion by single or multiple perfused human islets can be measured in a (commercially available) perifusion system using a well-characterized ELISA for human insulin. These
investigations document optimal criteria for pulse detection from human islets using two statistically independent methods for pulse detection, cluster analysis and a multi-parameter deconvolution method. The analyses further define the sensitivity of the two techniques for pulse detection. The deconvolution technique effectively quantitated insulin secretion rates, and accurately identified the number, location and magnitude of insulin secretory bursts exceeding 20% of baseline. Deconvolution provided a similar estimate of the frequency of insulin pulses whether islets were perifused singly or in groups. Cluster analysis provided a reasonable estimate of insulin pulse number and location so long as the magnitude of the oscillations was 40% or more above baseline, and that groups rather than single islets were studied. Our appraisal highlights a cautionary note inasmuch as multiple assay replicates per se and/or a perifusion system may generate spurious pulses in insulin concentration series (protocol 1a or protocol 1b) in the absence of genuine biological secretory bursts. As the human insulin assay used in the current studies has a lower coefficient of variation and is more sensitive and specific than available rodent insulin assays or prior human insulin assays, these problems could be expected to be magnified when studying rodent islets, or human islets using conventional radioimmunoassays.

Application of the newly validated techniques in human islets (protocol 3) provided some interesting physiological insights. The frequency of detected insulin pulses from human islets was one pulse ~7 minutes, which is comparable to that observed in vivo when sampling directly from the portal vein in dogs or humans (18,21,22). Sampling from the systemic circulation in humans with conventional immunoassays initially yielded a lower pulse frequency (e.g. interpulse interval of 15-20 minutes) (4,17). However, subsequent systemic sampling studies have measured insulin concentration(s) by ELISA and applied
deconvolution analysis validated for application in vivo (19,24,27). These methods disclosed insulin pulse frequency comparable to that observed in the current and earlier studies of single perfused islets. In fact examination of figure 2 reveals how small amplitude pulses compared to the baseline present in the systemic circulation in vivo are vulnerable to an underestimate of the pulse frequency, particularly when discrete peak detection analysis is used. In the current studies, the observed frequency of pulsatile insulin release by deconvolution was comparable whether islets were perfused as single islets or in groups of islets. The frequency is consistent with prior studies of perfused groups of islets (6,12). Using rodent islets, it has been necessary to perifuse groups of islets to allow adequate detection of insulin release or enlarged single mouse islets from genetically obese mice (2,3).

The current studies are also consistent with prior in vivo (and in vitro) studies, which suggest that regulation of the rate of insulin secretion is predominately achieved through modulation of the pulsatile component of secretion. Here we report that stimulation of insulin secretion in individual human islets by an increased concentration of glucose or potassium is achieved mechanistically via the specific enhancement of the insulin pulse mass with no change in pulse frequency. Measurement of pulsatile insulin in vivo by sampling from the portal vein to avoid hepatic insulin extraction reveals that almost all insulin is secreted in the pulsatile mode (18,22). However, in the present studies in vitro examination of the insulin released by perifused human islets reveals a that the proportion of insulin secreted in discrete bursts is ~30-50% (or more). This apparent difference might be due to the inefficiency of insulin delivery from the islet β- cells in vitro into the perifusion system compared with the delivery of insulin to the portal vein in vivo. In the
latter context, β-cells discharge insulin vesicles into capillaries, which have a relatively high blood flow moving past all the β-cells prior to the collection of this blood into the pancreatic veins and the portal vein. In contrast, in vitro, the capillaries in the islets are lost and insulin secreted by β-cells (located predominately in the core of the islet) would be delivered into spaces within the islet, from which it could diffuse to the biogel in which the islet is embedded and then to the buffer to be delivered from the perifusion apparatus. Insulin might transiently adhere to biogel beads and the walls of the collecting vessels prior to appearing in the eluate. These factors would be expected to result in a damping of the waveform of the insulin released by the islet, which would contribute to the predicted effect of underestimating the proportion of insulin released in discrete bursts. Moreover, the removal of the islets from the pancreatic neural network and other paracrine influences may influence the secretory behavior of these cell clusters.

In summary, in the present study we have established the conditions for reliable detection of pulsatile insulin secretion from human islets in an in vitro perifusion system. Application of the system to human islets reveals that in general perifused islets behave remarkably alike islets in vivo. Thus, we confirm that insulin is released in pulses at an interval of ~6-8 minutes and that regulation of insulin secretion (stimulation by increased potassium or glucose concentrations) is achieved predominately by amplification of the magnitude of these insulin bursts. The minimum proportion of insulin released by isolated islets that can be confidently ascribed to discrete insulin bursts is lower that which we have previously observed in vivo. This could reflect some degree of anatomic disruption of the islet and/or decreased resolution of pulses per se. The present system should allow more detailed examination of the factors that generate and regulate discrete insulin bursts by human islets.
References


Chapter 5

Diazoxide attenuates glucose-induced defects in first phase and pulsatile insulin secretion in human pancreatic islets
Introduction

Diabetes mellitus is characterised by hyperglycaemia and impaired glucose mediated insulin secretion (1). It has been shown that chronic hyperglycaemia per se leads to a reversible defect in insulin secretion. One explanation for this is a decrease in β cell sensitivity to hyperglycaemia (desensitisation) (2). Another explanation is that chronically increased demand on insulin stores leads to a relative decrease in available insulin vesicles for secretion. Insulin stores have been shown to be depleted in animal models of chronic hyperglycaemia (3), islet cultures at high glucose (4) and in pancreatic extracts from patients with type 2 diabetes mellitus (5). If depleted insulin stores contribute to decreased insulin secretion under conditions of chronic hyperglycaemia then temporary inhibition of insulin secretion would be expected to lead to at least partial restoration of these stores as well as insulin secretion.

In animal models of chronic hyperglycaemia inhibition of insulin secretion by diazoxide has been shown to restore insulin stores and first phase and second phase insulin secretion (6). In human studies one-week treatment with diazoxide in patients with type 2 diabetes restored first phase insulin secretion (7) and overnight inhibition of insulin secretion with somatostatin restored first phase insulin secretion and pulsatile insulin secretion (8).

In the present studies we examined insulin content and insulin secretion (in static incubations and islet perifusions) to address the following hypotheses. First, we hypothesised that prolonged (96 hour) incubation of islets at physiological increased glucose concentration (11 mmol/l) leads to decreased islet insulin content and decreased insulin secretion. Second, we hypothesise that inhibition of insulin secretion under these conditions (diazoxide) leads to a relative preservation of islet insulin stores and secretion. If
the latter hypothesis is confirmed the implication is that at least a component of defective glucose mediated insulin secretion following prolonged exposure of islets to increased glucose concentrations is attributable to depletion of insulin stores. Thirdly, that the magnitude of insulin pulses secreted by an islet is related to the islet insulin stores. Finally, that the increment in insulin secretion following prior diazoxide treatment is achieved through amplification of the magnitude of insulin pulses.

**Methods and materials**

**Overall experimental design**

The purpose of the current studies was to determine the impact of transient inhibition of insulin secretion on the subsequent pulsatile and first phase insulin secretion by human islets. To accomplish this, batches of 5-20 islets were incubated for periods of 1 hr, 24 hrs and 96 hrs in the presence or absence of 0.5 mmol/L diazoxide or 500 nmol/L somatostatin prior to measurement of first and second phase insulin secretion. In order to measure pulsatile insulin secretion and first phase insulin release islets were perifused at a stimulating glucose concentration of 20 mmol/L perifusate collected at one minute intervals for 40 minutes. Following islet perifusion the islets were salvaged to measure the total islet insulin content. This provided the opportunity to examine the relationship between changes in islet insulin content (achieved during the static incubations) and subsequent first phase and pulsatile insulin secretion.

**Islet isolation**

Human islets were isolated from pancreases retrieved from 3 heart-beating organ donors by the department of surgery at the University of Leicester. No donor had a history of diabetes
mellitus. After isolation, islets were cultured in RPMI 1640 medium containing 5.5 mmol/l glucose, 2 mmol/L glutamine, 10% fetal calf serum, 100 U/ml benzylpenicillin and 0.1 mg/ml streptomycin. Culture period in Leicester varied between 1-5 days before islets were sent to Edinburgh. After arrival at this laboratory, islets were transferred to new RPMI 1640 medium (same composition as above) and cultured for at least 2 days before experiments were performed. Time elapsed from islet isolation in Leicester to the start of actual experiment was 9±2.2 days (range 3-13).

It was important to have islets of comparable insulin content at the start of each protocol arm. We therefore sized islets (by microscopy) and distributed islets to each group for subsequent study so that they included comparable proportions of small (80-160 μm) intermediate (160-240 μm) and large (240-300 μm) islets.

*Experimental protocol*

(1) *Static incubation* (Table 1)

Islets were cultured at 37 °C with an atmosphere of 5% CO₂/95% O₂. The islets were incubated for 3 different time periods (1 hr, 24 hrs and 96 hrs) in 3 mls of RPMI 1640 culture medium. In the 1-hr and 24-hr incubation groups, batches of 20 islets were cultured in 5.5 mmol/L and 11 mmol/L glucose in presence of 0.5mmol/L diazoxide, 500 nmol/L somatostatin or control RPMI 1640 medium. In the 96-hr incubation group, batches of 5-10 islets were cultured at 11 mmol/L glucose with 0.5 mmol/L diazoxide or control RPMI 1640 medium.
TABLE 1

Conditions of static incubation with corresponding duration of culture, glucose concentration and presence or absence of diazoxide (DZ) or somatostatin (SMS) or control (CON)

<table>
<thead>
<tr>
<th>Incubation period (hr)</th>
<th>Glucose (mmol/L)</th>
<th>SMS 500 nmol/L</th>
<th>DZ 0.5 mmol/L</th>
<th>CON RPMI 1640</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.5</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>24</td>
<td>5.5</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>24</td>
<td>11</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>96</td>
<td>11</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>
At the end of each incubation period, the culture medium was collected for analysis of insulin concentration to allow calculation of insulin secretion during the static incubation. The islets were transferred immediately to the perifusion protocol.

(2) Islet perifusion

After the static incubation period, islets were periperfused using a temperature-controlled multichamber perifusion system (ACUSYST-S; Cellex Biosciences, Minneapolis, MN). Islets were removed from the static incubation media, and immediately suspended in Bio-Gel P-2 beads (Bio-Rad, Hercules, CA) and placed in aliquots of 5-20 islets 500 uL perifusion chambers. The perifusion system consisted of a multi-channel peristaltic pump which delivered perfusate through 6 parallel tubing sets via a heat exchanger and six perifusion chambers. The peristaltic pump drew perfusate at a rate of 0.3 ml/min and delivered it through the heat exchanger where it was warmed to 37 °C and gassed with 95% O₂. From the heat exchanger, the perfusate was delivered to the perifusion chambers containing the human islets. The perifusion protocol consisted of exposing the islets to Kreb’s Ringer bicarbonate buffer perfusate (115 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, 5 mmol/L NaOH) supplemented with 0.2% human serum albumin, 1 mol/L HEPES, 2 mmol/L glutamine, 100 U/ml benzylpenicillin, 0.1 mg/ml streptomycin and 20 mmol/l glucose for 45 minutes. Effluent perfusate was collected at 1-minute interval and stored at -20 °C and subsequently analysed for insulin concentration.
Measurement of islet insulin content post-perfusion

At the end of perifusion period, islets were recovered from the perifusion chamber for the measurement of its insulin content as previously described (9). The islets were washed in RPMI 1640 and lysed with 600 μL ice-cold lysis buffer (50 mM Hepes, 0.1% (vol/vol) Triton X-100, 1 μM PMSF, 10 μM E-64, 10 μM pepstatin A, 10 μM TLCK, 100 μM leupeptin: pH 8.0). After sonication (25W for 40 s) and centrifugation (12000 g for 5 mins), the resultant supernatants were stored at -20 °C and subsequently analysed for insulin concentration and thus islet insulin content.

Assays

Glucose. Glucose concentrations were measured by glucose oxidation method using a glucose analyser (Beckmann, Palo Alto, CA).

Insulin. Insulin concentrations were measured in duplicate by two-site immunospecific insulin ELISA assay as previously described (10). The assay uses two monoclonal murine antibodies (Novo Nordisk, Bagsvaerd, Denmark) specific for insulin. There is no cross-reactivity with proinsulin and split 32,33 and 31,32 proinsulins. Sensitivity of the assay is 5 pmol/l and the detection range is 5-2000 pmol/l. The intra-assay and inter-assay CVs ranged from 2.0-2.3% and 3.7-4.5% respectively.

Proinsulin. Proinsulin concentrations were measured in duplicate by two-site ELISA assay (11). The assay uses two monoclonal antibodies specific for proinsulin (Novo Nordisk, Bagsvaerd, Denmark). The detection limit is 0.05–20 pmol/L. Interassay CVs were 4.7% at
a median of 2.3 pmol/L, 6.7% at 5.1 pmol/L and 8.7% at 10 pmol/L. There is no cross
reactivity with human insulin and C-peptide.

Calculations and statistical analysis
All results are expressed as mean ± SEM. First phase insulin secretion was defined by the
mean secretion rate in the first 9 minutes after exposure to 20 mmol/l glucose and the
second phase secretion was defined as the mean secretion rate between min 10 and min 45
of the perifusion experiment. The total insulin secretion during perifusion was calculated as
the sum of first and second phase secretion.

Islet insulin content at the end of the perifusion experiment was calculated by:

[Equation 1]: Islet insulin content postperifusion (fmol/islet) = insulin content in lysis
buffer (fmol)/number of islets.

Islet insulin content at the end of the static incubation (and just prior to the perifusion) was
calculated as follows.

[Equation 2]: Islet insulin content post static incubation (fmol/islet) = Islet insulin content
postperifusion (fmol/islet) + [insulin released during perifusion (fmol)/ number of islets].

Islet insulin content prior to static incubation was then calculated by:

[Equation 3]: Islet insulin content pre static incubation (fmol/islet) = Islet insulin content
post static incubation (fmol/islet) + [insulin released during static incubation (fmol)/ number
of islets].
Pulsatile insulin secretion was quantified by both cluster analysis and deconvolution methods using parameters specifically developed and validated for the present apparatus with human islets and the current assay (12). Please refer to chapter 4 of this thesis for full details.

Statistical comparison was made by use of either the unpaired Student’s t test, analysis of variance or regression analysis. A p value of p<0.05 was considered to be significant.

**Results**

*Insulin secretion during static incubations.*

There was no significant difference in islet size (by design) or islet insulin content prior to inclusion of the islets in any of the protocols (Table 2). During static incubation of human islets, insulin was released into the media under all conditions of study. As expected, insulin release increased at 5.5 vs 11 mmol/l glucose concentrations (1 hour; 13.8 ± 7.2 vs 40.4 ± 14.9 fmol/islet/hr, 24 hours; 2.6 ± 0.38 vs 20.7 ± 5 fmol/islet/hr, p<0.05). Islets cultured at a glucose concentration of 11 mmol/l for 96 hrs had a progressive but borderline significant reduction in insulin release rate (e.g. control islets at 1 vs 24 vs 96 hrs, 40.4±14.9 vs 20.7±5 vs 7.3±1.6 fmol/islet/min p=0.07 ANOVA). As expected, at physiological increased glucose concentrations both diazoxide and somatostatin inhibited insulin secretion. Thus, when islets were cultured for 24 hours at 11 mmol/L glucose, inclusion of either diazoxide or somatostatin in the medium led to a decreased rate of insulin release (SMS vs CON, 3±0.6 vs 20.7±5 fmol/islet/hr, p<0.05 and DZ vs CON, 3.2±0.8 vs 20.7±5 fmol/islet/hr, p<0.05). Similarly, when diazoxide was added to culture medium over 96
<table>
<thead>
<tr>
<th></th>
<th>SMS</th>
<th>DZ</th>
<th>CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr at 5.5 mM glucose</td>
<td>955.7±207.6</td>
<td>1016.2±205.6</td>
<td>735.6±242.2</td>
</tr>
<tr>
<td>1 hr at 11 mM glucose</td>
<td>1013±262.4</td>
<td>1234.9±310.7</td>
<td>804.6±255.5</td>
</tr>
<tr>
<td>24 hrs at 5.5 mM glucose</td>
<td>1571.4±437.6</td>
<td>929.6±110.5</td>
<td>1231.1±161.3</td>
</tr>
<tr>
<td>24 hrs at 11 mM glucose</td>
<td>1788.4±274.3</td>
<td>1559.8±207.8</td>
<td>1947.8±565.2</td>
</tr>
<tr>
<td>96 hrs at 11 mM glucose</td>
<td>1052.4±261.8</td>
<td>1919.7±205.2</td>
<td></td>
</tr>
</tbody>
</table>
hours at a glucose concentration of 11 mmol/l, insulin secretion was decreased (figure 1a) (DZ vs CON, 3.5±0.75 vs 7.3±1.6 fmol/islet/hr, p<0.05). In contrast, no effect of either diazoxide or somatostatin applied for 1 hour was detectable on the mean insulin secretion rate (at glucose 5.5 or 11.0 mmol/l) presumably reflecting the short period of study. Of interest, when islets were incubated for 24 hours diazoxide and somatostatin inhibited insulin secretion (p<0.05) at physiological high (11 mmol/l) but not basal (5 mmol/l) glucose concentrations. In conclusion these data indicate that in the present studies the islets responded appropriately by increasing insulin secretion in response to increased glucose concentrations, and that this increased secretion rate was partially inhibited by either somatostatin or diazoxide treatment at the physiological high glucose concentrations. These conditions therefore allow us to test the posed hypotheses.

Islet insulin content after static incubation (Table 3)

Neither somatostatin nor diazoxide had any measurable impact on insulin content in islets cultured for either 1 or 24 hours at 5.5 mmol/l glucose. These observations were consistent with the lack of a detectable impact of somatostatin or diazoxide on insulin secretion rates by islets after they had been cultured under these conditions.

Islets cultured for 96 hours at 11 mmol/L glucose had a significantly reduced insulin content compared to islets cultured at the same glucose concentration for 1 or 24 hours (control islets, 1 vs 96 hrs, 762.5±250.8 vs 216.9±62.4 fmol/islet, p<0.05, 24 vs 96 hrs, 1451.7±462.6 vs 216.9±62.4 fmol/islet, p<0.05). This indicated that between 24 and 96 hours the rate of insulin release was greater than its rate of synthesis, thus, depleting islet insulin stores. However, application of diazoxide to islets cultured for 96 hours at 11 mmol/l
Insulin secretion rate

96-hr incubation at 11 mmol/L glucose. Insulin secretion rate into culture medium was reduced from pancreatic islets incubated in diazoxide. This demonstrated the inhibitory effects of diazoxide on glucose-induced insulin secretion.
**TABLE 3**

Islet insulin content after static incubation. Islet insulin content is expressed in the unit of fmol/islet. Results are expressed as mean ± SEM of 4-6 experiments.

<table>
<thead>
<tr>
<th></th>
<th>SMS</th>
<th>DZ</th>
<th>CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr at 5.5 mM glucose</td>
<td>955.7±207.6</td>
<td>1016.2±205</td>
<td>735.6±242.4</td>
</tr>
<tr>
<td>1 hr at 11 mM glucose</td>
<td>987.1±252.8</td>
<td>1200.5±299.5</td>
<td>762.5±250.8</td>
</tr>
<tr>
<td>24 hrs at 5.5 mM glucose</td>
<td>1561.9±441.1</td>
<td>913.8±105.7</td>
<td>1197.8±154.7</td>
</tr>
<tr>
<td>24 hrs at 11 mM glucose</td>
<td>1716.2±263.6</td>
<td>1482.9±198.6</td>
<td>1451.7±462.6</td>
</tr>
<tr>
<td>96 hrs at 11 mM glucose</td>
<td></td>
<td>712.8±190*</td>
<td>216.9±62.4</td>
</tr>
</tbody>
</table>

* p<0.05 vs CON in 96 hr incubation at 11 mmol/L glucose
glucose led to a relative preservation of insulin content compared to control islets (figure 1b) (DZ vs CON, 712.8±190 vs 216.9±62.4 fmol/islet, p<0.05). This observation is consistent with the observed decreased rate of insulin secretion by islets exposed to diazoxide during the 96-hour studies.

First and second phase insulin release during perifusion

Islet insulin content predicted first or second phase insulin secretion. There was no difference in the first or second phase insulin release as a result of prior incubation of islets with diazoxide for 1 or 24 hours, neither of which was associated with a change in insulin stores. However, incubation of islets at 11 mmol/L glucose with diazoxide for 96 hours preserved islet insulin content relative to controls (see above) and also led to increased first phase, (DZ vs CON, 6.0±0.97 vs 3.7±0.6 fmol/min/islet, p<0.05) and second phase insulin secretion (DZ vs CON, 2.1±0.4 vs 1.2±0.2 fmol/min/islet, p<0.05) (figure 1c, 1d and figure 2). There was a strong correlation between islet insulin stores at the start of the perifusion and first phase (r=0.40, p<0.001) and second phase (r=0.60, p<0.001) during perifusion (figure 3).

Pulsatile insulin release

Insulin pulses were detected in 66 of the 72 islet perfusions. The mean pulse interval was 8.9 ± 0.4 mins by cluster analysis and 6.6 ± 0.4 mins by deconvolution. The magnitude of insulin pulses correlated with islet insulin content (r=0.5, p<0.001) whilst there was no such correlation for inter-pulse interval (r=−0.06, p=0.6) (figure 4). Treatment with diazoxide had no effect on the insulin pulse interval (deconvolution, DZ vs CON, 6.7 ± 0.5 vs 6.7 ± 0.5)
Fig 1b.

96-hr incubation at 11 mmol/L glucose. At the end of the static incubation period, the islet insulin content was higher in pancreatic islets incubated in diazoxide. This demonstrated the protective effect of diazoxide against hyperglycaemia-induced chronic overstimulation.
First phase secretion

96-hr incubation at 11 mmol/L glucose. First phase insulin secretion is higher from pancreatic islets incubated in diazoxide.

Fig 1c.
Second phase secretion

96-hr incubation at 11 mmol/L glucose. Second phase insulin secretion is higher from pancreatic islets incubated in diazoxide.
Fig 2.

96-hr incubation at 11 mmol/L glucose. Insulin concentration profile during islet perifusion demonstrating first and second phase secretion.
Fig 3.
Correlation between islet insulin content and first and second phase secretion. There is positive correlation between islet insulin content and both first and second phase secretion.
Fig 4.

Correlation between islet insulin content and magnitude of insulin pulses and inter-pulse interval. There is positive correlation with the magnitude of insulin pulses but not with inter-pulse interval.
mins, p=0.5 and cluster, DZ vs CON, 8.0 ± 1.5 vs 8.8 ± 1.4 mins, p=0.3). The increment in the first and second phase insulin secretion observed in islets cultured at physiological increased glucose concentration (11 mmol/L) with diazoxide were accompanied by an increase in insulin secretion rate (deconvolution, DZ vs CON, 12.0 ± 2.3 vs 6.9 ± 1.1 fmol/min, p<0.05), pulse magnitude (deconvolution, DZ vs CON, 13.3 ± 1.6 vs 9.0 ± 1.6 pmol/L, p<0.05) and islet insulin content at the start of perifusion (DZ vs CON, 712.8±190 vs 216.9±62.4 fmol/islet, p<0.05).

*Islet insulin content after perifusion* (Table 4)

Islet insulin content was higher in islets cultured with diazoxide for 96 hours at 11 mmol/L glucose (DZ vs CON, 579.7±168 vs 138.8±60.8 fmol/islet, p<0.05). There was no difference in islet insulin content following islet perifusion attributable to SMS or DZ at either 1 or 24 hours incubations at 5.5 or 11 mmol/l glucose.

*Proinsulin/insulin ratio (96 hr incubation)*

(1) *Static incubation*

The inhibitory effect of diazoxide on insulin secretion (DZ vs CON, 339.6 ± 72.2 vs 702.8 ± 154 fmol/islet/96hrs, p<0.05) was also reflected in the proinsulin release (DZ vs CON, 2.5 ± 0.7 vs 4.7 ± 1.2 fmol/islet/96hrs, p=0.06). Since the amount of insulin and proinsulin secreted was reduced approximately 2-fold by diazoxide, there was no difference in the PI/I ratio (DZ vs CON, 0.70±0.1% vs 0.67±0.1%, p=0.4).
TABLE 4

Islet insulin content after perfusion. Islet insulin content is expressed in the unit of fmol/islet. Results are expressed as the mean ± SEM of 4-6 experiments.

<table>
<thead>
<tr>
<th>Time at Glucose</th>
<th>SMS</th>
<th>DZ</th>
<th>CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr at 5.5 mM</td>
<td>667.1±186.9</td>
<td>837.9±177.5</td>
<td>529.6±138.4</td>
</tr>
<tr>
<td>1 hr at 11 mM</td>
<td>686.1±130.2</td>
<td>891.7±265.8</td>
<td>392.3±229</td>
</tr>
<tr>
<td>24 hrs at 5.5 mM</td>
<td>1297.2±397.2</td>
<td>681.9±142.1</td>
<td>976.5±153</td>
</tr>
<tr>
<td>24 hrs at 11 mM</td>
<td>1398.4±209.2</td>
<td>1173.8±222.3</td>
<td>1103.6±399.4</td>
</tr>
<tr>
<td>96 hrs at 11 mM</td>
<td></td>
<td>579.7±168 *</td>
<td>138.8±60.8</td>
</tr>
</tbody>
</table>

*p<0.05 vs CON in 96hr incubation at 11 mM glucose
(2) Islet perifusion

During perifusion, there was no difference in the mean proinsulin concentration released from islets treated with diazoxide (DZ vs CON, 0.16 ± 0.03 vs 0.20 ± 0.06 pmol/L, p=0.3). In contrast, the mean insulin concentration secreted was higher from these islets (DZ vs CON, 72.8 ± 12.8 vs 29.5 ± 3 pmol/L, p<0.01). Therefore, diazoxide significantly lowered the perifusion PI/I ratio by approximately 71% (DZ vs CON, 0.2 ± 0.03% vs 0.7 ± 0.2%, p<0.05) (figure 5a).

(3) Islet content post-perifusion

Following perifusion, the islet insulin content was reduced in the control islets (DZ vs CON, 579.7 ± 168 vs 138.8 ± 60.8 fmol/islet, p<0.05). In contrast, there was no significant difference in the islet proinsulin content (DZ vs CON, 4.7 ± 1.2 vs 3.5 ± 1.4 fmol/islet, p=0.2). Therefore, diazoxide significantly reduced the islet content PI/I ratio following perifusion by approximately 65% (DZ vs CON, 0.85 ± 0.04% vs 2.4 ± 0.3%, p<0.001) (figure 5b).

Discussion

In the present study we report that incubation of human islets at a glucose concentration typically present in patients with Type 2 diabetes mellitus (11 mmol/l) for 96 hours results in depletion of insulin stores. We confirm that this depletion is decreased when insulin secretion is partially inhibited by concurrent incubation of the islets with diazoxide and this treatment leads to enhanced insulin secretion (first phase, second phase and pulsatile) in response to glucose stimulation.
Fig 5a.

Perifusion PI/I ratio. Following 96-hr incubation at 11 mmol/L glucose, diazoxide reduced the PI/I ratio by 71%.
Fig 5b.
Post-perifusion islet content PI/I ratio. Following 96-hr incubation at 11 mmol/L glucose, diazoxide reduced the PI/I ratio by 65%.
It has previously been reported that human islets cultured at supra-physiological glucose concentrations have depleted insulin stores (4,13,14). This study demonstrates that incubating non-diabetic human islets for 96 hrs at a physiological high glucose concentration (11 mmol/L) leads to depletion of islet insulin stores and associated impairment of both first and second phase insulin secretion. These data support the concept that one of the mechanisms by which prolonged hyperglycaemia leads to β-cell dysfunction is through the depletion of the available insulin stores. If this concept is valid then enhancement of insulin stores in islets of patients with Type 2 diabetes would be expected to result in improved insulin secretion. Hales et al (7) reported improvement in first phase insulin secretion in a group of patients with Type 2 diabetes who were treated with diazoxide for one week. Normalising plasma glucose concentrations by diet, insulin and sulphonylurea treatments also led to improved endogenous insulin secretion (15). More recently, it was shown that patients with Type 2 diabetes who had temporary overnight inhibition of insulin secretion by somatostatin infusion restored their first phase insulin secretion and pulsatile insulin release (8). In vitro studies have demonstrated that the improvement in insulin secretion was associated with an increase in islet insulin content. 90% pancreatectomised rats treated with diazoxide had improved insulin release during pancreatic perfusion and this was associated with higher pancreatic insulin content (6). Human pancreatic islets cultured at 27 mmol/L glucose for 48 hours had impaired glucose-induced insulin secretion and depleted islet insulin stores and both these defects were partially restored with diazoxide (13). It appears that the imbalance between insulin release and synthesis resulting in depletion of insulin stores in conditions of chronic hyperglycaemia can lead to β-cell dysfunction and that by ‘resting’ the β-cell from chronic
hyperlstimulation can partly reverse this defect through the enhancement of the islet insulin content.

The relative proportion of proinsulin level (PI/I ratio) is elevated in subjects with Type 2 diabetes (16,17). Here we report that the PI/I ratio observed during dynamic islet perifusion and in the post-perifusion islet content are both decreased in islets cultured in diazoxide at high physiological glucose concentration (11 mmol/L). The aetiology of elevated PI/I ratio has been postulated to be the consequence of intrinsic β-cell defect (in the processing of proinsulin) (18,19) or the release of immature (proinsulin-rich) granules which are accumulated in higher proportion than the mature (insulin-rich) granules within the islet following hyperglycaemia-induced hyperstimulation of the β-cell (20,21). The results of the present study support the latter hypothesis. Diazoxide, which blocks glucose-induced insulin secretion and opposes the effect of hyperstimulation, causes a higher proportion of insulin to be accumulated within the islet whilst the absolute amount of proinsulin remains unchanged. This results in the reduction of the relative proportion of intra-islet proinsulin (PI/I ratio) observed here. This effect is also seen in the dynamic perifusion where a higher proportion of insulin is released relative to proinsulin from islets incubated in diazoxide at high physiological glucose concentration, presumably reflecting the mobilisation of these mature insulin-rich granules.

Depletion of the pancreatic and islet insulin content has been shown to be associated with defective glucose-induced insulin secretion (4,6,13,14). Further support for the role of insulin content on insulin release was provided in an autopsy study which showed that subjects with Type 2 diabetes have at least 50% reduction in their pancreatic insulin content
In the present study, we report a positive correlation between islet insulin content and first phase and second phase insulin secretion. Pulsatile insulin secretion is also modulated by islet insulin content. By employing the validated optimal criteria for pulse detection for both cluster and deconvolution methods to analyse pulsatile insulin secretion from human pancreatic islets (12), we report that the magnitude of insulin pulses is positively correlated with the quantity of islet insulin stores whilst there is no such relationship for the inter-pulse interval and that, the restoration of insulin secretion rate and magnitude of insulin pulses in islets incubated in diazoxide were accompanied by increment in islet insulin stores. This present study therefore yields further support for the role of islet insulin stores in the modulation of the magnitude of insulin secretion and reaffirms that the regulation of pulsatile insulin secretion is achieved through modulation of its pulsatile component at the level of human pancreatic islets.

Is depleted insulin store as a consequence of β-cell hyperstimulation the sole explanation for impaired insulin secretion observed in hyperglycaemic condition? The insight to this question was provided by an observation made in this study. In islets cultured at physiological high glucose concentration (11 mmol/L), the rate of insulin secretion into the incubation medium declined from culture duration of 1 hour to 24 hours without any significant change in the islet insulin content. Hence, depletion of islet insulin store per se could not be the sole cause of impaired insulin secretion. High glucose condition or other unknown factor(s) can impose deleterious effect on the machinery involved in glucose-induced insulin secretion. Various hypotheses has been proposed such as defects in insulin gene transcription (14,22), decreased insulin mRNA expression with associated proinsulin biosynthesis (22,23), defective glucose metabolism (24), defective function of ATP
sensitive K+ channel (25) and accumulation of glycogen within β-cell (26). Thus, it is plausible that one or more of these factors could also contribute to β-cell dysfunction and hence, restoring islet insulin content only remedies one of the factors causing defective glucose-induced insulin secretion. This could explain the partial restoration of first phase insulin secretion following a period of β-cell rest in patients with Type 2 diabetes (27,28).

More recently, it has been demonstrated that diazoxide only partially restore insulin content in human islets cultured in high glucose conditions (27 mmol/L) (13).

Another interesting observation is that diazoxide confers beneficial effect on insulin secretion only in condition which results in depletion of islet insulin stores. In islets cultured at high physiological glucose concentration for 24 hours, pre-treatment with diazoxide and somatostatin had no positive effect on first phase and second phase insulin secretion. However, this is in contrast to the islets cultured at high physiological glucose concentration for 96 hours which resulted in significant depletion of insulin stores and pre-treatment with diazoxide in this group of islets led to improvement in insulin secretion. This observation is in agreement with those of Leahy (6) and Grill (29) who noted in diabetic rat models the need for reduced insulin content to see the full protective effect of diazoxide on insulin secretion. Therefore, it is logical to conclude that the beneficial effect of β-cell rest on insulin secretion only occurs in conditions that result in excessive insulin release outstripping its synthesis capacity.

What are the implications of the protective effects conferred by diazoxide on insulin secretion? Firstly, it strengthens the hypothesis that chronic β-cell stimulation can lead to
defective insulin release and this could be partly explained by depletion in islet insulin content. Secondly, it provides a potential method to treat type 2 diabetes therapeutically. For example, the pancreas could be rested overnight during which time requirement for insulin secretion is lowest allowing the pancreatic insulin stores to accumulate and to be released in greater quantities in response to meal ingestion during daytime. Ideally, a pharmacological agent without significant side effect(s) and with short half-life might be suitable. The short half-life will allow shorter time for the drug to exert its effect and also for its inhibitory action to disappear fairly rapidly to allow the release of insulin. Thirdly, β-cell rest conferred by diazoxide has been shown to prevent the progression to diabetes in fatty diabetic zucker rats (30). This has implication that not only that β-cell rest could be beneficial in the treatment of established diabetes but also in reducing or preventing the risk of progressing to clinically overt diabetes especially in high-risk individuals ie siblings of diabetic patient, first-degree relatives or subjects with metabolic risk factors. Fourthly, chronic administration of diazoxide has been shown to preserve endogenous insulin secretion in newly diagnosed type 1 diabetic patients (31). This provides further support for the beneficial effects of β-cell rest and also demonstrates that it is also effective in type 1 diabetes which has a different (autoimmune) aetiology from type 2 diabetes.

In summary, we have shown that culturing non-diabetic human islets for 96 hours at physiological high glucose (11 mmol/L) typically observed in Type 2 diabetes subjects leads to depletion of islet insulin stores and defective insulin secretion. Incubating these islets with diazoxide improves the glucose-induced insulin secretion (first phase, second phase and pulsatile) and this is associated with restoration of islet insulin stores. Islet insulin stores correlates positively not only with first phase and second phase insulin secretion but
also with the magnitude of insulin pulses. Finally, the regulation of pulsatile insulin secretion from human islets is achieved predominantly through selective amplification of the pulse magnitude whilst its frequency remains unchanged.

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Chapter 6

Conclusions
Following the completion of these projects, a few interesting observations and conclusions were made.

Pulsatile insulin secretion is regulated via selective amplification of the secretory burst mass. It is secreted almost exclusively in discrete bursts \textit{in vivo}. However, in perfused islets, the proportion of insulin secreted in discrete bursts is less possibly as a consequence of anatomical disruption of the islet and/or decreased pulse resolution.

The frequency of insulin pulses in human portal circulation is similar to those observed in the portal circulation of dogs and \textit{in vitro} isolated perfused islets. Moreover, it is also comparable to those observed in systemic circulation \textit{in vivo} when insulin concentration was assayed by using highly sensitive immunoassay and analysed by validated deconvolution method. This resolved the discrepancy in the literature regarding the frequency of pulsatile insulin secretion \textit{in vivo}. Furthermore, it implies that the activity of the pancreatic pacemaker is remarkably consistent and stable in both \textit{in vivo} and \textit{in vitro} conditions.

Another important observation is that the liver is exposed to large oscillations of insulin concentration and the modulation of hepatic insulin clearance by the magnitude of insulin pulses in portal vein. Physiological studies which examined the effects of pulsatile insulin delivery on hepatic insulin action utilised oscillations of insulin concentration that were less than those observed in the portal vein. This may have implications on the interpretation of the physiological actions of pulsatile insulin delivery on the liver.
The importance of distinguishing the oscillations in insulin concentration as a consequence of model and assay noise from true pulsatile insulin secretion was highlighted in the validation study. This is particularly important in the analysis and interpretation of the oscillations in insulin concentration from isolated perifused islets where the absolute insulin concentration levels can be low.

Chronic hyperglycaemia at levels observed in Type 2 diabetes can result in defective insulin secretion and depleted islet insulin stores. ‘Resting’ the β cells (with diazoxide) can restore the first and second phase and pulsatile insulin secretion. This was associated with increased islet insulin content. The importance of islet insulin content on insulin secretion was emphasised by the observation of the excellent correlation between islet insulin content and the magnitude of insulin secretion (first and second phase and magnitude of insulin pulses). This strengthens the hypothesis that impaired glucose-induced insulin secretion including pulsatile secretion is at least partially due to depleted insulin stores as a consequence of hyperglycaemia-induced chronic overstimulation of β-cells.

In conclusion, the dynamics of insulin secretion are important as well as the absolute secretion and that the abnormalities in these dynamics may be partly related to β-cell insulin stores which can be manipulated by transient β-cell rest. Since the discovery that insulin is secreted in pulsatile manner, its importance has been increasingly recognised in the pathophysiology of diabetes mellitus. It is hoped that the results emanating from this MD thesis might contribute in some way to the understanding of the physiology of insulin secretion and the potential therapeutic application in clinical diabetes.