THE ETIOLOGY AND NATURAL HISTORY OF TYPE 2 DIABETES

by

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BSc, Simon Fraser University, 1998

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Abstract

Type 2 diabetes is a complex disorder characterized by progressive defects in nearly every aspect of metabolic regulation. Despite this complexity, traditional *in vivo* methodologies have limited experimental examination to a small number of metabolic indices at one or two points in time. As a result the etiology and natural history of this disease remain unclear and much debated. This thesis takes a two pronged approach to this problem. First, a mathematical model is developed to incorporate experimental data from different sources into an integrated representation of metabolic regulation. Bifurcation and simulation analysis of this model are used to investigate mechanisms of metabolic regulation as well as the pathogenesis of type 2 diabetes. Second, new experimental methodologies are developed that greatly improve the practicality of estimating several key metabolic indices *in vivo*. Applying these methodologies to animal models of type 2 diabetes allowed us to perform a fully dynamic and integrative analysis of the pathogenesis of type 2 diabetes in two commonly used animal models. Overall, data from this thesis suggests that the etiology of type 2 diabetes lies in two distinct abnormalities; rapid development of insulin resistance coupled to impaired β-cell mass adaptation.

Keywords:

Type 2 diabetes, etiology, pathogenesis, beta cell mass, beta cell function, insulin secretory defects, insulin sensitivity, insulin resistance, mathematical modeling, bifurcation, nonlinear dynamics

To my mother.

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Table of Contents

List of Tables

List of Figures

INTRODUCTION

Type 2 diabetes is a debilitating disease defined by high concentrations of glucose in the blood (hyperglycemia). Chronic hyperglycemia has been shown to cause several microvascular and neuronal complications that often result in blindness, kidney and heart failure, stroke, neuropathies and limb amputation [1, 2]. At present, type 2 diabetes affects over 150 million people world wide and is one of the leading causes of morbidity and mortality in Western society [3, 4]. In addition type 2 diabetes is estimated to cost the world economy hundreds of billions of dollars per year (over 13 billion in Canada alone) [5, 6, 7]. Although a significant burden already exists, epidemiological studies suggest that increasing obesity in Western societies, aging of the baby boomer population and Westernization of traditional cultures will double the cost (human and economic) of type 2 diabetes over the next 20 years [3, 4].

Blood glucose levels are determined largely by the rates of hepatic glucose production and muscle glucose uptake. While each of these processes is controlled by complex interactions of several metabolites and hormones, insulin is widely considered to be the dominant controller. Hyperinsulinemia drives blood glucose levels down via inhibition of hepatic glucose production and stimulation of muscle glucose uptake. Insulin's actions at the liver occur via inhibition of glycogenolysis and stimulation of glycogen synthesis. Although there have been some reports suggesting that insulin also regulates hepatic gluconeogenesis, this pathway appears to be largely substrate driven [8]. At the muscle, insulin signaling stimulates the translocation of GLUT4 containing intracellular vesicles to the cell membrane increasing the passive flux of glucose from the interstitial space into the cytoplasm. Interstitial glucose is quickly converted into glucose-6-phosphate, via hexokinase, then converted into glycogen or metabolized. Insulin also has an indirect effect on glucose uptake and production via inhibition of free fatty acid release at the adipocyte and triglyceride production at the liver.

Blood insulin levels are predominantly determined by the rates of insulin secretion, though there is increasing evidence to suggest decreased insulin clearance may contribute to the chronic hyperinsulinemia associated with obesity [9, 10, 11]. Glucose is the dominant regulator of insulin secretion. Glucose is transported into the cell by GLUT2 transporters, converted to glucose-6-phosphate by glucokinase, then rapidly metabolized. Increases in blood glucose levels lead to increased rates of glucose metabolism and elevated levels of cystolic ATP. This increase in the ATP to ADP ratio stimulates oscillations in membrane potential and intracellular calcium levels that result in translocation and binding of intracellular vesicles to the cell membrane, releasing insulin into the interstitial space. The magnitude of insulin release has been shown to be determined by levels of other metabolites (including amino acids and lipids) and hormones (including glucagon like peptide-1 [GLP-1] and gastric inhibitory peptide [GIP]). As described above, this increase in blood insulin levels drives glucose levels down and, as a result, decreases the stimulus for insulin secretion. Together the pancreas, liver and muscle constitute a negative feedback loop regulating blood glucose levels.

Obesity is associated with reduced insulin action at the muscle and liver (insulin resistance). The mechanisms linking obesity to insulin resistance have been widely debated. Several lines of study have suggested that the dyslipidemia of obesity impairs glucose uptake by acting as a competitive fuel that inhibits mitochondrial glucose metabolism (Randle cycle) [12]. This would lead to accumulation of the metabolic intermediates of glucose metabolism, including glucose-6-phosphate, that would inhibit flux through GLUT4 transporters. However, insulin resistance appears to be associated with abnormal insulin-induced translocation of GLUT4 rather than reduced flux through these membrane transporters. The observation of a strong correlation between muscle triglyceride levels and insulin sensitivity suggests that lipid accumulation, (rather than metabolism), interferes with insulin signalling [13]. Subsequent research has suggested that insufficient post prandial lipid buffering at the adipocyte leads to excessive lipid exposure and uptake by organs such as the muscle, liver, and pancreas [14]. Intermediates in the conversion of fatty acids to intracellular triglycerides are postulated to reduce insulin

signalling via competitive inhibition of insulin receptor substrates (IRS-1, and 2) binding to phosphoinositide 3 (PI3) kinase in muscle and liver cells [15].

While dyslipidemia has been widely touted as a primary cause of insulin secretory defects, the majority of obese individuals display elevated fasting and acute response insulin levels such that net insulin action remains unchanged [16, 17]. These observations have led several researchers to postulate the existence of a feedback loop connecting peripheral insulin action to pancreatic β-cell adaptation. However, the signals for and mechanisms of β-cell adaptation remain unresolved. For years it was assumed that the pancreatic mass was static and that β-cell adaptation occurred via β-cell hypersecretion. However, there is growing evidence to suggest the contrary. It is now well established that the pancreatic β-cell mass is dynamic and responsive to changes in insulin demand. Glucose infusion, partial pancreatectomy, and transgenic mouse studies have shown β-cell replication, death and neogenesis to be responsive to increased insulin demand *in vivo* [18, 19, 20]. Histological studies have shown β-cell mass to be elevated in obese humans and animal models [21, 22]. While several studies have demonstrated acute adaptation of β-cell secretory capacity [23, 24], there is increasing evidence to suggest that these adaptations are not sustainable on a time scale of years. Several studies have provided support for the concept of β-cell exhaustion suggesting that β-cell hyper-stimulation causes the secretory defect observed in type 2 diabetes [25, 26]. Also, several, but not all, *in vitro* studies have shown β-cell secretory capacity to be similar in islets from lean and obese donors [22, 27, 28, 29]. We are unaware of any study that has reported the dynamics *in vivo* of β-cell function and β-cell mass together during the development of obesity or type 2 diabetes.

Several metabolic and hormonal signals have been proposed as the signal connecting insulin resistance to β-cell adaptation, including glucose, lipids, insulin, and leptin [18, 30, 31, 32, 33, 34]. Of these signals glucose has received the most attention. Hyperglycemia has been shown to induce changes in β-cell function, mass, replication, death and neogenesis *in vitro* and *in vivo* [35, 36, 37, 38, 39]. *In vitro* studies have demonstrated clear nonlinear relationships between glycemia and both β-cell replication and death rates [38, 39]. These

results suggest that moderate hyperglycemia stimulates expansion of the β-cell mass while extreme hyperglycemia causes contraction of β-cell mass. While there is some *in vivo* data to support the concept of glucose induced β-cell mass adaptation, the relationship between glycemia and β-cell replication/death rates have not clearly delineated *in vivo* [18, 30, 40]. Also several experimental models of increased insulin demand, including obesity, have shown compensatory hyperinsulinemia in the absence of any detectable change in blood glucose levels [23, 41]. Thus the physiological role of glucose in β-cell adaptation to obesity remains to be clarified.

Unlike normal obesity, type 2 diabetes is characterized by insufficient insulin levels. While insulin sensitivity is reduced dramatically, often by more than 90%, blood insulin levels are similar or only slightly elevated relative to lean insulin sensitive individuals [42, 43]. Cross-sectional studies have shown the progression of type 2 diabetes to be characterized by a nonlinear "Starling" curve of blood insulin levels (insulin levels are elevated in pre-diabetes then decrease as the disease progresses from mild to severe) [43]. Although the root cause of progressive β-cell failure remains unresolved, there is increasing evidence to suggest that hyperglycemia and dyslipidemia contribute significantly to this process [16]. In addition, hyperglycemia and dyslipidemia have been shown to induce insulin resistance *in vivo* [13, 44]. However, it should be noted that establishment of near normoglycemia via aggressive treatment of type 2 diabetes in the UKPDS did not prevent the progression of this disease [45]. Nonetheless, it is generally accepted that poor metabolic control contributes significantly to the progression of type 2 diabetes.

The concepts of glucotoxicity and lipotoxicity have placed increased focus on the events surrounding the initiation of hyperglycemia and dyslipidemia. This time period is characterized by decreasing insulin sensitivity and increasing blood insulin levels. Thus it is likely that the initiation of hyperglycemia arises from abnormally rapid decreases in insulin sensitivity or slow β-cell compensation. It is possible that hyperglycemia results from an inability to sustain "normal" levels of hyperinsulinemia (β-cell exhaustion).

While there is some evidence to suggest that the early stages of type 2 diabetes are characterized by excessive levels of insulin resistance, most studies have found insulin resistance does not differ substantially between subjects with pre to mild type 2 diabetes vs. normoglycemic obese controls [42, 46]. Similarly, some studies have shown insulin secretory defects to be the earliest defects observed in the pathogenesis of type 2 diabetes; other studies have suggested that insulin secretory defects occur secondary to the development of hyperglycemia and dyslipidemia [47, 48]. Further, it is unclear if this proposed early defect represent abnormal β-cell function, β-cell mass or both. Much of the uncertainty about the early stages of type 2 diabetes arises naturally from the fact that these primary metabolic indices are generally studied in isolation from one another [49]. While researchers have begun to recognize the need for integrative analysis, the progressive nature of this disease also makes the need for dynamic analysis self evident.

The relative lack of integrative and dynamic studies of the natural history of type 2 diabetes is likely due to methodological limitations. β-cell mass and replication rates can only be determined from histological analysis of pancreatic tissue. At present there are no well accepted methodologies for quantifying rates of β-cell apoptosis and neogenesis *ex vivo*. Thus, it is not possible to determine the relative contributions of β-cell function and β-cell mass to indices of *in vivo* insulin secretory capacity. Insulin sensitivity is regularly quantified *in vivo* in humans and large animals with the hyperinsulinemic clamp or minimal model analysis of intravenous glucose tolerance test data. However, these methodologies are considered too cumbersome and expensive for large scale longitudinal studies. Thus, while recent advances in genetic and molecular biology have vastly expanded the capacity and practicality of measuring dozens of indices at several points in time *in vitro*, present *in vivo* methodologies render integrative and dynamic analysis of metabolic regulation expensive and impractical. As a result our understanding of the genetic and molecular characteristics of metabolic pathways has grown extensively while fundamental questions such as the mechanisms linking insulin resistance to β-cell adaptation or the relative contributions of insulin resistance, insulin secretory defects and reduced β-cell mass to the pathogenesis of type 2 diabetes remain largely unresolved.

This thesis focuses on integrative and dynamic analysis of metabolic regulation. Three general approaches are taken. First, a mathematical model is utilized to integrate data from different sources and investigate the mechanistic links connecting various metabolic processes. Second, this mathematical model is converted into a data analysis tool allowing for indirect estimation of central metabolic indices in small animal models. This allows for fully integrative and dynamic analysis of metabolic processes *in vivo*. Finally, a 24 hour hyperglycemic clamp protocol is developed to fully quantify the relationship between glycemia and β-cell mass adaptation *in vivo*. While several questions are addressed in this thesis there are three central question: 1) is glucose the signal connecting insulin resistance to β-cell mass adaptation, 2) what are the relative contributions of β-cell mass and β-cell function to the hyperinsulinemia of type 2 diabetes, and 3) what are the relative contributions of insulin resistance, insulin secretory defects and reduced β-cell mass to the pathogenesis of type 2 diabetes in leptin receptor deficient rats.

Chapter 1 describes the development of a mathematical model of glucose induced β-cell mass dynamics based largely on the available *in vitro* data. This model is then incorporated into a minimal mathematical model of the glucose regulatory system. This model is used to address two questions. The first question is how does the model respond to the development of insulin resistance? In other words, is glucose a viable signal connecting insulin resistance to β-cell mass adaptation? The second question is what abnormalities or perturbations need to be incorporated into the model to convert it from a redundant regulatory system to one that displays the pronounced and progressive hyperglycemia that characterized type 2 diabetes?

Chapter 2 describes the conversion of the β-cell mass, insulin and glucose (βIG) model from chapter 1 into a data analysis tool that allows for indirect estimation of insulin sensitivity, β-cell function and net neogenesis in small laboratory animals. Model predictions are validated via application to chronic glucose infusion data that was

previously collected in our laboratory. This experimental protocol was chosen as a useful validation experiment as it is associated with complex adaptations in each of the indices of interest and there is useful data in the literature to compare our predictions against.

Chapter 3 describes the utilization of the βIG model methodology to fully quantify the natural history of insulin sensitivity, β-cell mass, and β-cell function during the development of obesity and type 2 diabetes. The family of Zucker Fatty rats were utilized in this chapter as they are commonly used animal models and provide several interesting comparisons. Zucker Fatty (ZF) rats possess a leptin receptor defect that makes them obese and overtly insulin resistance, however, these animals remain normoglycemic. Inbreeding of ZF rats led to the development of Zucker Diabetic Fatty (ZDF) rats. Male ZDF rats spontaneously develop hyperglycemia and display several similarities to human type 2 diabetes. Female ZDF rats only become hyperglycemic when placed on a high fat diet. Thus by using male ZF, male ZDF, low fat fed female ZDF and high fat fed ZDF rats we were able to investigate the mechanisms of genetically induced diabetes (male ZDF vs. male ZF), diet induced diabetes (low vs. high fat fed female ZDF rats) and sexual dimorphisms in metabolic control (male ZDF vs. low fat fed female ZDF rats). Full quantification of the natural history of insulin sensitivity, β-cell function and β-cell mass in these animal models allows us to directly assess both the contributions of β-cell mass and function to obesity, as well as the contributions of insulin sensitivity, β-cell function and β-cell mass to the pathogenesis of hyperglycemia.

Finally, Chapter 4 described the development and application of a 24 hour hyperglycemic clamp protocol that is utilized to fully quantify the relationship between glycemia and β-cell mass adaptation *in vivo*. β-cell mass, replication, death, and neogenesis were quantified at five glucose levels spanning the physiological range (basal, 15, 20, 25 and 35 mmol). Quantification of the dose response relationship between glycemia and β-cell mass adaptation *in vivo* should provide insight into the relationship between glycemia and β-cell mass adaptation *in vivo*.

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CHAPTER 1: THE βIG MODEL.

A Model of β-Cell Mass, Insulin, and Glucose Kinetics: Pathways to Diabetes

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Abstract

Diabetes is a disease of the glucose regulatory system that is associated with increased morbidity and early mortality. The primary variables of this system are β–cell mass, plasma insulin concentrations, and plasma glucose concentrations. Existing mathematical models of glucose regulation incorporate at most two of these variables. Here we develop a novel model of β-cell mass, insulin, and glucose dynamics. This model consists of a system of three nonlinear ordinary differential equations, which contain fast and slow subsystems. We analyze the local and global behavior of this system. For normal parameter values, this β–cell mass, Insulin, Glucose (βIG) model has two stable and one unstable steady state solution. When parameter values are allowed to vary, three characteristic pathways to diabetes are revealed: a regulated hyperglycemia pathway, a bifurcation pathway, and a dynamic hyperglycemia pathway.

1. Introduction

Diabetes mellitus is a disease of the glucose regulatory system characterized by fasting and/or postprandial hyperglycemia [1]. There are two major classifications of diabetes based on etiology. Type 1 diabetes (also referred to as juvenile onset or insulin-dependent diabetes) is due to an autoimmune attack on the insulin secreting β-cells. Type 2 diabetes (also referred to as adult onset or non-insulin-dependent diabetes) is associated with a deficit in the mass of β-cells [2], reduced insulin secretion and resistance to the action of insulin [1]. The relative contribution and interaction of these defects in the pathogenesis of this disease remain to be clarified [3, 4].

Blood glucose levels are regulated by two negative feedback loops. In the short term, hyperglycemia stimulates a rapid increase in insulin release from the pancreatic β cells. The associated increase in blood insulin levels cause increased glucose uptake and decreased glucose production leading to a reduction in blood glucose [5]. Recent evidence suggests that chronic hyperglycemia may contribute to a second negative feedback loop by increasing the mass of insulin secreting β-cells [6], through changes in the rates of β-cell replication [7, 8] and death [9, 10]. An increased β-cell mass represents an increased capacity for insulin secretion which, in turn, would lead to a decrease in blood glucose. Type 2 diabetes has been associated with defects in components of both the short-term and chronic negative feedback loops.

Although type 2 diabetes is associated with insulin resistance, insulin secretory defects, and insufficient β-cell mass, each of these defects can also be found in people without diabetes. Insulin-stimulated glucose disposal is reduced by 50-100% in patients with type 2 diabetes as compared to non-diabetic controls [11]. However, insulin resistance of a similar magnitude also has been documented in many non-diabetic individuals including obese subjects, or during pregnancy, puberty, and aging [11]. Thus, normoglycemia can be maintained in subjects with insulin resistance via increases in blood insulin levels. Defects of insulin secretion have been demonstrated in some people with type 2 diabetes [12]. Even more severe defects in insulin secretion are present in patients with type 1

diabetes following islet transplantation, when normoglycemia is maintained in the absence of exogenous insulin treatment [13]. This suggests that glucose homeostasis can be maintained despite significant loss of β-cell function when an individual has normal insulin sensitivity. Kloppel *et al.* observed that β-cell mass is reduced by 40-50% in patients with type 2 diabetes when compared with weight matched non-diabetic subjects [2]. In comparison, approximately 80-90% of the β-cell mass is lost before the onset of hyperglycemia in individuals who develop type 1 diabetes suggesting that a greater β-cell mass is required in the presence of insulin resistance [2]. This is also consistent with the observation of a 43% higher β-cell mass in normoglycemic subjects with insulin resistance due to obesity [2].

Although these data suggest that multiple defects are required for the onset of type 2 diabetes, it is unclear if these defects have a single causal origin or if they occur independently [4]. Experimental induction of insulin resistance using either high fat feeding [14], glucocorticoid administration [15], or genetically induced obesity [16] has been shown to cause type 2 diabetes under certain circumstances. This supports the hypotheses that insulin resistance can cause β-cell defects, and hence diabetes, either by overworking the β-cells (β-cell exhaustion) [17, 18] or by toxic effects of hyperglycemia on the β-cells (glucose toxicity) [19]. However, the existence of normoglycemia in humans and animals highly resistant to insulin suggests independent defects in insulin sensitivity and β-cell function are required for type 2 diabetes [5, 20]. Finally, hyperglycemia is known to induce insulin resistance [21]. This supports the hypothesis that a primary insulin secretory defect that causes hyperglycemia could lead to insulin resistance and diabetes via increased glucose levels.

Mathematical modeling in diabetes research has focused predominately on the dynamics of a single variable, usually blood glucose or insulin levels, on a time scale measured in minutes [22, 23, 24]. Generally, these models are used as tools for measuring either rates (such as glucose production and uptake rates or insulin secretion and clearance rates) or sensitivities (such as insulin sensitivity, glucose effectiveness, or the sensitivity of insulin

secretion rates to glucose). Two model-based studies have examined coupled glucose and insulin dynamics [5, 25]. In each of these studies, multiple parameter changes, representing multiple physiological defects, were required to simulate the glucose and insulin dynamics observed in humans with diabetes. However, neither of these efforts incorporated β-cell mass dynamics. Here, we take an integrative mathematical approach towards developing a deeper understanding of the normal and pathological behavior of the glucose regulatory system. We develop a mathematical model of β-cell mass dynamics as a component of a coupled model of β-cell mass, insulin, and glucose dynamics. This model allows us to examine the normal behavior of the system and to investigate the effects of a single defect or a combination of defects on the behavior of the system as a whole. In doing so, we find three characteristic pathways to the diabetic state: regulated hyperglycemia, bifurcation and dynamic hyperglycemia.

2. Model Development

2.1. Glucose Dynamics

In the post absorptive state, glucose is released into the blood by the liver and kidneys, removed from the interstitial fluid by all the cells of the body, and distributed into many physiological compartments (e.g. arterial blood, venous blood, cerebral spinal fluid, interstitial fluid). In spite of the spatial complexity of the glucose distribution [26], Steele suggested that the rates of glucose production and uptake could be calculated with isotope dilution techniques and a one-compartment model of glucose kinetics [23]. Allsop *et al.* and Radziuk *et al.* validated the use of Steele's single compartment equation for near steady-state glucose dynamics [27, 28]. However, when glucose or tracer concentrations are changing rapidly, (on a time scale of minutes), a single compartment model is not adequate [29]. Consistent with these observations is the demonstration by Bergman *et al.* that a single compartment can be used to model glucose kinetics following an intravenous glucose bolus, provided the rapid mixing phase (~10 min) is not considered [22]. Together, these studies suggest that a single compartment model is appropriate when glucose kinetics are relatively slow. Since this model is primarily concerned with the evolution of

fasting blood glucose levels over a time scale of days to years, glucose dynamics are modeled with a single-compartment mass balance equation,

$$dG/dt = P - U \quad (1)$$

where G is the concentration of glucose in the blood (measured in mg dl^{-1}), t is time (measured in days), and P and U represent the rates of endogenous glucose production and uptake from the extracellular fluid, respectively (measured in mg d^{-1}). We normalize P and U by the volume of glucose distribution to obtain proper units (mg dl^{-1} d^{-1}).

The rates of glucose production and uptake depend on blood glucose and insulin levels. These relationships have been defined experimentally with the glucose clamp technique, which allows for the measurement of glucose production and uptake rates at various steady-state blood glucose and insulin levels [5]. At constant insulin levels, glucose production decreases while uptake increases, both linearly with respect to glucose levels [30]. The slopes of these linear dependences are the "glucose effectiveness" parameters. Hyperinsulinemic clamp studies have shown that the glucose effectiveness parameters are linearly related to blood insulin levels. The slope of the glucose effectiveness vs. insulin curve is referred to as insulin sensitivity. Thus,

$$\text{Production} = P_0 - (E_{G0P} + S_{IP}I)G, \quad (2)$$

$$\text{Uptake} = U_0 + (E_{G0U}\, S_{IU}I)G, \quad (3)$$

where P_0 and U_0 are the rates of glucose production and uptake at zero glucose (mg dl^{-1} d^{-1}), E_{G0P} and E_{G0U} (d^{-1}) are glucose effectiveness at zero insulin for production and uptake, S_{IP}, S_{IU} (μU^{-1} ml d^{-1}) are insulin sensitivity for production and uptake, and I represents blood insulin concentrations (μU ml^{-1}). Substituting eqns (2) and (3) into eqn (1), we arrive at

$$dG/dt = R_0 - (E_{G0} + S_I I)G \quad (4)$$

where R_0 (= P_0 - U_0) is the net rate of production at zero glucose, E_{G0} (= E_{G0P} + E_{G0U}) is the total glucose effectiveness at zero insulin, and S_I (= S_{IP} + S_{IU}) is the total insulin sensitivity.

2.2. Insulin Dynamics

Insulin is secreted by pancreatic β-cells, cleared by the liver, kidneys, and insulin receptors, and distributed into several compartments (e.g. portal vein, peripheral blood, interstitial fluid). Our main concern is the long-time evolution of fasting insulin levels in peripheral blood. Since the dynamics of fasting insulin levels on this time scale are slow, we use a single-compartment equation given by

$$dI/dt = \text{Secretion} - \text{Clearance} \tag{5}$$

where Secretion and Clearance are rates normalized by insulin's volume of distribution ($\mu U\ ml^{-1}\ d^{-1}$).

The rate of insulin clearance is proportional to blood insulin levels when the system is near steady state [31]. We note that several single-compartment models which incorporate proportional insulin clearance have simulated relatively fast and complex insulin dynamics successfully [24, 32]. Given the relatively slow dynamics in the present model, we assume

$$\text{Clearance} = kI \tag{6}$$

where k is a clearance constant which represents the combined insulin uptake at the liver, kidneys, and insulin receptors (d^{-1}).

The rate of insulin secretion from pancreatic tissue has been shown to be sigmoidally related to glucose concentration [33]. However, the relative contribution of β-cell recruitment and cellular insulin secretion rates has not been quantified [34]. Studies by Schuit *et al.* and Bosco & Meda demonstrated a sigmoidal relationship between the percentage of insulin secreting β-cells and the glucose level [35, 36]. Schuit *et al.* demonstrated a sigmoidal relationship between glucose levels and the percentage of β-cells containing newly synthesized proinsulin, while Bosco & Meda showed that the majority of insulin released from the β-cell mass comes from cells which are actively synthesizing proinsulin. Studies analyzing individual β-cell and islet electrical activity [37, 38], granulation [39], and insulin secretion [34] have shown that the rate of secretion from

individual β-cells varies with glucose, but a functional relationship has yet to be identified.

The *in vitro* relationship determined by Malaisse *et al.* (1967) as well as the successful use of sigmoid insulin secretion rates in existing models (Cobelli *et al.*, 1980, Rudenski *et al.*, 1991) indicate that the net insulin secretion rate can be modeled as sigmoidally related to glucose levels [33, 40, 41]. Hence, we assume

$$\text{Secretion} = \beta\sigma G^2/(\alpha + G^2) \tag{7}$$

where β is the mass of pancreatic β-cells (measured in mg). All β-cells are assumed to secrete insulin at the same maximal rate σ (μU ml^{-1} d^{-1}), and $G^2/(\alpha + G^2)$ is a Hill function with coefficient 2 that describes a sigmoid ranging from 0 to 1 which reaches half its maximum at $G = \alpha^{1/2}$. Substituting (6) and (7) into (5), we arrive at the equation governing insulin kinetics,

$$dI/dt = \beta\sigma G^2/(\alpha + G^2) - kI. \tag{8}$$

2.3. β-Cell Mass Dynamics

Despite a complex distribution of pancreatic β-cells throughout the pancreas, β-cell mass dynamics have been successfully quantified with a single-compartment model [42]

$$d\beta/dt = \text{Formation} - \text{Loss}, \tag{9}$$

where Formation and Loss represent the rates at which β-cell mass is added to or removed from the population, respectively.

New β-cells can be formed by proliferation of existing β-cells, neogenesis (proliferation and differentiation) from stem cells, and transdifferentiation of other cells. Presently, it is not possible to quantify rates of neogenesis and transdifferentiation; however, indirect calculations suggest that they make a negligible contribution to β-cell mass dynamics except during development and in response to extreme physiological or chemically induced trauma [42, 43, 44]. For these reasons, neogenesis and transdifferentiation are not incorporated into the present model, and Formation is assumed to be equal to Replication.

In vitro studies show that the percentage of β-cells undergoing replication varies as a nonlinear function of glucose level in the medium [7, 8]. Replication rates for β-cells increase with increasing glucose levels; however, at extreme hyperglycemia, β–cell replication may be reduced. We modeled this behavior with a simple second-degree polynomial (patterned after logistic growth),

$$\text{Replication} = (r_{1r}G - r_{2r}G^2)\beta \tag{10}$$

where r_{1r} (mg^{-1} dl d^{-1}) and r_{2r} (mg^{-2} dl^2 d^{-1}) are rate constants.

Cells can be lost from the β-cell mass by apoptosis (regulated cell death), necrosis (unregulated cell death), or possibly transdifferentiation into other types of endocrine cells. For the model presented here, the rate of transdifferentiation is assumed to be negligible. Therefore, β-cell Loss is assumed to equal β-cell Death, which has been shown to vary nonlinearly with glucose [9, 10]. Increasing the glucose level from 0 to ~11 mM in the medium surrounding cultured β-cells, reduces the β-cell death rate. Above 11 mM glucose, cell death either remained low or increased. We have modeled this behavior with a simple second degree polynomial.

$$\text{Death} = (d_0 - r_{1a}G + r_{2a}G^2)\beta \tag{11}$$

where d_0 (d^{-1}) is a death rate at zero glucose and r_{1a} (mg^{-1} dl d^{-1}) and r_{2a} (mg^{-2} dl^2 d^{-1}) are constants. Substituting (10) and (11) into (9), we arrive at the equation for β-cell mass dynamics,

$$d\beta/dt = (-d_0 + r_1G - r_2G^2)\beta \tag{12}$$

where r_1 ($= r_{1r} + r_{1a}$) and r_2 ($= r_{2r} + r_{2a}$) are constants.

In summary, the βIG model is comprised of equations (4), (8), and (12). Parameter values assumed for the normal human physiological state are given in Table 1. Below, we examine both the behavior of solutions of the βIG model in the phase space of the variables using the standard parameter values, (Table 1), and the change in geometrical structure of the solutions in the phase space as the parameter values are varied. Finally, we postulate some plausible pathways that lead from a normal physiological state to a pathological diabetic state.

Table 1- 1 Normal Parameter Values

S_I	0.72	ml μU^{-1} day^{-1}	Finegood, 1997
E_{G0}	1.44	day^{-1}	Bergman *et al.*, 1981 Finegood, 1997
R_0	864	mg dl^{-1} day^{-1}	Bergman *et al.*, 1981 Finegood, 1997
σ	43.2	μU ml^{-1} day^{-1}	Bergman *et al.*, 1981 Malaisse *et al.*, 1967 Toffolo *et al.*, 1980
α	20000	mg^2 dl^{-2}	Malaisse *et al.*, 1967
k	432	day^{-1}	Toffolo *et al.*, 1980
d_0	0.06	day^{-1}	Bergman *et al.*, 1981 Imamura *et al.*, 1988 Finegood *et al.*, 1995
r_1	0.84 10^{-3}	mg^{-1} dl day^{-1}	Bergman *et al.*, 1981 Imamura *et al.*, 1988 Finegood *et al.*, 1995
r_2	0.24 10^{-5}	mg^{-2} dl^2 day^{-1}	Bergman *et al.*, 1981 Imamura *et al.*, 1988 Finegood *et al.*, 1995

3. Model Behavior

Putting the model into dimensionless form (appendix) the model can be decomposed into fast (G, I) and slow (β) subsystems and the number of parameters can be reduced from 9 to 8 (reduction occurs in the glucose equation). The fast subsystem describes acute changes in glucose and insulin levels on a time scale of minutes while the slow subsystem describes evolution of the β-cell mass on a time scale of days. We will examine the behavior of these subsystems independently and then analyze the system as a whole. In the following, we will continue to use dimensional variables.

Due to its slow dynamics, β-cell mass will be treated as a parameter when analyzing the fast subsystem. Plotting the nullclines ($dG/dt = 0$ and $dI/dt = 0$) for a given normal β-cell mass value, examining the solution trajectories, and performing a local and global stability analysis of the steady state solution shows that the fast subsystem has a single

globally attracting stable spiral (Figure 1-1A). The effect of β-cell mass on the behavior of the fast subsystem is demonstrated in Figure 1-1B. Each β-cell mass value is associated with a single globally attracting fixed point; however, as β increases, this fixed point shifts to lower glucose levels and higher insulin levels. In summary, beginning at any initial condition, blood glucose and insulin levels will follow a damped oscillation in time settling down to a steady state which is dependent on the β-cell mass.

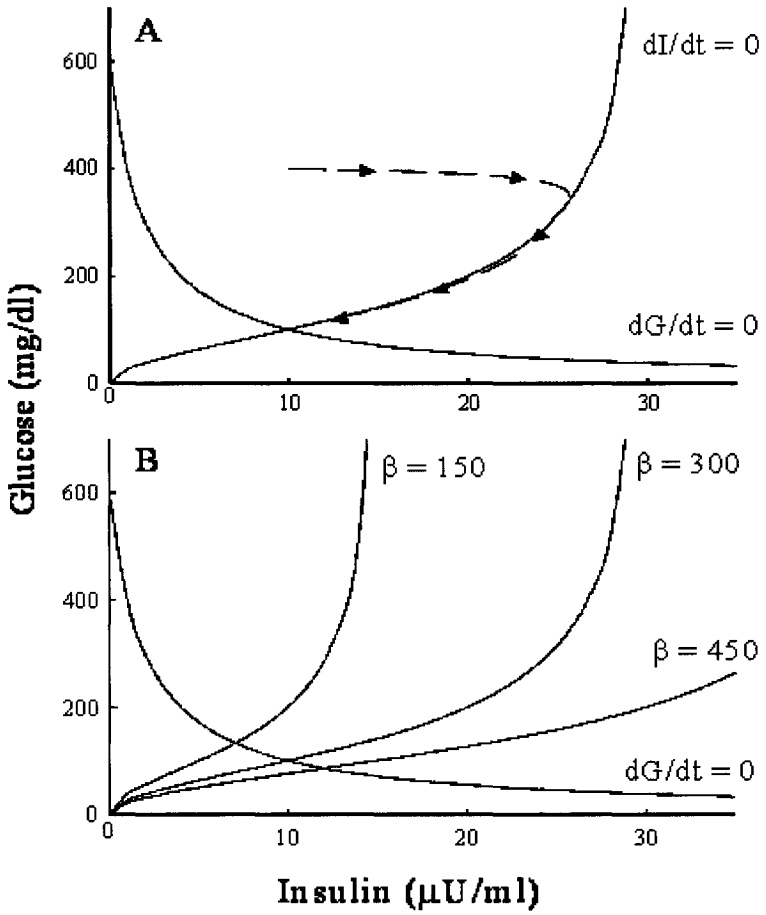

Figure 1- 1 Global behavior of the fast subsystem. (A) shows the glucose nullcline, the insulin nullcline, and a simulation of the response of the fast subsystem to a bolus injection of glucose (dashed line), with the β-cell mass is held constant at 300 mg/dl. Stability analysis shows that the fixed point, located at the intersection of the glucose and insulin nullclines, is a globally attractive stable spiral. (B) shows the glucose nullcline and three insulin nullclines, for β = 150, 300, and 450. As the β-cell mass increases, the fixed point of the fast subsystem moves to lower glucose and higher insulin levels, but the global behavior remains unchanged

To study the slow subsystem, we assume that the fast subsystem is at a steady state, and changes in the β-cell mass slowly shift that steady state. The one-dimensional slow subsystem, (12), has three steady state solutions, which we index by $\beta = 0$, $G = 100$, and $G = 250$. These three steady states will be referred to as the pathological, physiological, and unstable steady states respectively.

The behavior of the slow subsystem is best described using a plot of the β-cell replication and death rates, (10) and (11), as functions of glucose levels as shown in Figure 1-2. This graph can be divided into three zones of behavior. For Zone I (hypoglycemia), death rates are greater than replication rates and the β-cell mass decreases. Since a decreasing β-cell mass causes glucose levels to rise, this zone regulates hypoglycemia back to the physiological steady state. In Zone II (mild hyperglycemia), replication rates are greater than death rates, thereby causing the β-cell mass to grow. Since increasing β-cell mass causes glucose levels to decrease, this zone regulates mild hyperglycemia back to the physiological steady state also. Taken together, Zones I and II constitute a basin of physiological regulation. However, in Zone III (extreme hyperglycemia), death rates exceed replication rates, thereby driving glucose levels even higher. This positive feedback loop drives the system to the pathological steady state corresponding to zero β-cell mass and extreme hyperglycemia. For this reason, Zone III is referred to as a basin of dysregulation or a basin of pathological regulation. In summary, the slow subsystem has two stable steady states and an unstable steady state which divides the basins of attraction for the two stable points.

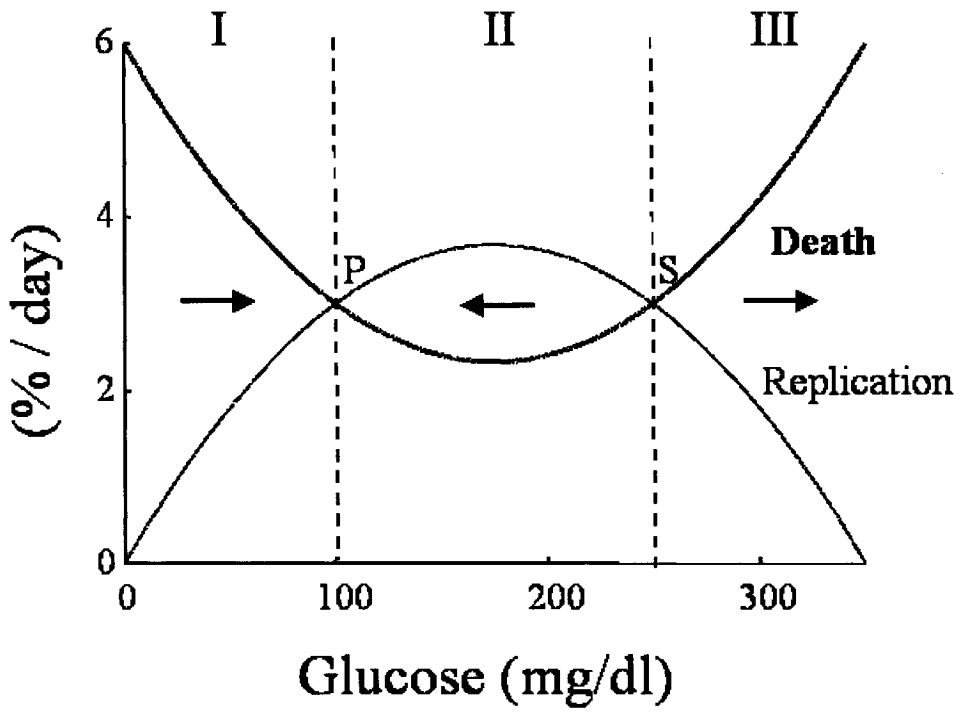

Figure 1- 2 Global behavior of the slow subsystem. Replication and death rates are plotted against blood glucose levels. These curves intersect at two nontrivial steady states, a physiological fixed point (P) and a saddle point (S). In addition, there is a pathological, trivial steady state at $\beta = 0$. In Zone I, death rates exceed replication rates, driving the β-cell mass down and causing steady state glucose levels to rise. In Zone II, replication rates exceed death rates driving β-cell mass up and blood glucose levels down. Zones I and II constitute a basin of attraction for the physiological fixed point (P). In Zone III, death exceeds replication, causing a decrease in β-cell mass and driving glucose levels even higher. This is a zone of pathological regulation, in which drives the system to the trivial steady state of zero β-cell mass.

Analyzing the system as a whole, the βIG model has three steady state solutions in (β, I, G): a stable spiral (300, 10, 100), a saddle point (37, 2.8, 250), and a stable node (0, 0, 600). These points correspond to the physiological fixed point, the saddle point, and the pathological fixed point of the slow subsystem, respectively. The two-dimensional glucose and insulin null surfaces are attractive and their intersection forms a one-dimensional slow manifold. All solutions move quickly along trajectories which are approximately perpendicular to the β-cell mass axis onto the slow manifold, and then

move along this slow manifold according to the β-cell mass dynamics. There are three β-cell mass null surfaces, namely, the planes defined by G = 100, 250 mg dl^{-1}, and β = 0 mg. A projection of the slow manifold and the β-cell mass nullsurfaces onto the G and β plane is given in Figure 1-3. For all points above G = 250 mg dl^{-1} and below G = 100 mg dl^{-1}, the β-cell mass is decreasing and for all points between these planes, the mass is increasing. The steady-state saddle point solution divides this slow manifold into two parts. In the upper part, β is decreasing and all solutions move towards the pathological fixed point, whereas in the lower part the β-cell mass dynamics drive all solutions towards the physiological fixed point (Figure 1-3). Due to the fast glucose and insulin dynamics, the global separatrix can be approximated by a plane that is perpendicular to the β-cell axis and passes through the saddle point. This plane then divides the phase space into two basins of attraction, one for the physiological fixed point and one for the pathological fixed point. The fast subsystem solution quickly comes to a steady state at the initial β-cell mass, then this steady-state solution is driven by the β-cell mass dynamics to either the physiological or pathological steady state.

The qualitative behavior of this model is consistent with observed behavior of the glucose regulatory system in response to changes in glucose concentration or β-cell mass. Increased plasma glucose, via an intravenous glucose injection, causes a rapid increase in plasma insulin followed by damped oscillations of both variables towards the pre-injection steady state [22]. Reduction of the β-cell mass, via administration of the β-cell toxin streptozotocin (STZ), generates transient hyperglycemia while the β-cell mass adapts[43]. Mild hyperglycemia, generated and maintained by a constant glucose infusion rate, also causes an increase in β-cell mass and plasma insulin that drive plasma glucose back towards pre-infusion levels [6]. However, when glucose levels are maintained at or above 250 mg/dl via a variable glucose infusion rate, the plasma insulin, as well as the rate of glucose infusion required to maintain this severe hyperglycemia, decrease over time [45]. All of these observations are consistent with the behavior of the βIG model.

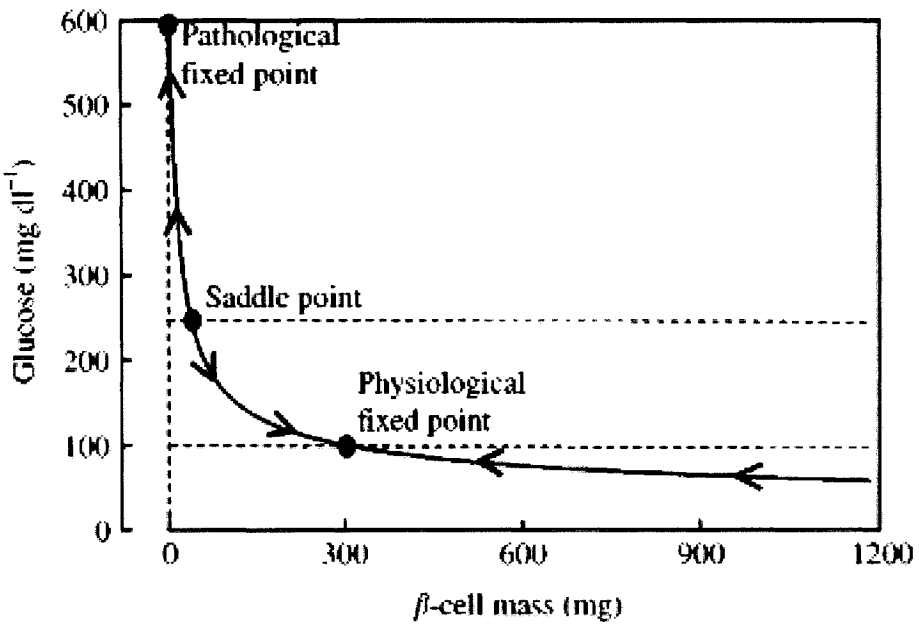

Figure 1- 3 Global behavior of the βIG model. Plotted here is a projection of the slow manifold and the β-cell mass null surfaces onto the G and β plane. In terms of the variables (β, I, G), the system has three steady states: physiological (300, 10, 100) and pathological (0, 0, 600) fixed points, and a saddle point at (37, 2.8, 250). All solutions travel on trajectories approximately perpendicular to the β-cell mass axis onto the slow manifold, then move along the slow manifold according to β-cell mass dynamics. The derivative dβ/dt is negative for all points below the G = 100 null surface, and above the G = 250 null surface and it is positive between the non-trivial β-cell mass null surfaces. The saddle point divides the slow manifold into two basins of attraction, one for each of the physiological and pathological fixed points: solid line = slow manifold; dashed line = β null surface.

4. Effects of Parameter Changes on Global Behavior

The number of nontrivial steady state solutions is determined by the parameters of the β-cell mass equation, while the position of these fixed points is determined by all nine dimensional parameters. The trivial steady state solution is fixed at zero β-cell mass and insulin, but has a steady state glucose level that is dependent on the glucose parameters (R_0, E_{G0}).

4.1. Defects In Glucose Dynamics

Defects in insulin sensitivity, represented by a reduced S_I, decrease the curvature of the glucose nullcline and thus the slow manifold. This results in a shifting of the

physiological and saddle fixed points to higher β-cell mass and insulin levels. For comparison, experimental reduction of insulin sensitivity via dexamethasone causes an increase in blood insulin and β-cell mass levels [15]. Defects in glucose dynamics represented by an increase in R_0 or a decrease in E_{G0} cause the glucose nullsurface, and the associated slow manifold to shift upwards. There is also a shifting of the physiological and saddle fixed points to higher β-cell mass and insulin levels, as well as a shifting of the pathological fixed point to a higher glucose level. Experimental augmentation of R_0, via continuous glucose infusion, has been shown to increase β-cell mass and plasma insulin levels [6, 46]. In summary, glucose dynamics have no effect on the model's physiologically regulated glucose level, but do affect the β-cell mass and insulin levels required to maintain normal fasting glucose levels as is consistent with experimental data.

4.2. Defects In Insulin Dynamics

An increase in the insulin clearance rate or a decrease in the insulin secretion rate per active β-cell, modeled by an increase in k or a decrease in σ, respectively, compresses the insulin nullcline in the direction of the insulin axis. This changes the curvature of the slow manifold, shifting the physiological and saddle points to higher β-cell mass values. This is similar to the observations of Curry *et al.* who have shown that the maximal insulin secretion rate per unit of β-cell mass decreases and β-cell mass increases with age in non-diabetic Fischer 344 rats [47]. Defects in the recruitment of β-cells from an inactive to an active state represented by an increase in α, stretches the insulin nullcline in the direction of the glucose axis, changing the curvature of the slow manifold. This also shifts the physiological and saddle points to higher β-cell mass values. It is not known if α, specifically, can be manipulated. In summary, insulin dynamics do not affect the glucose or insulin levels at the physiological fixed point, but do affect the β-cell mass required to maintain normal fasting glucose and insulin levels. These predictions are consistent with experimental data.

4.3. Defects In β-Cell Mass Dynamics

Defects in β-cell replication and/or death rates, represented by an increase in d_0 or r_2, or a decrease in r_1, cause the non-trivial β-cell mass nullsurfaces (G = 100 mg/dl and G = 250 mg/dl) to move closer together in the direction of the glucose axis. The physiological fixed point shifts to a point with higher glucose, lower insulin, and lower β-cell mass, while the saddle point shifts to a point with lower glucose, higher insulin, and higher β-cell mass, increasing the volume of the basin of pathological attraction. If a defect in β-cell mass dynamics progresses to the point where death rates exceed replication rates at all glucose levels, the number of steady state solutions is reduced to one, namely the globally attractive pathological steady state with zero β-cell mass. Although the relationship between plasma glucose levels and mild defects in β-cell replication and/or death rates has not been determined *in vivo*, an excessive increase in the β-cell death rate has been shown to drive β-cell mass towards zero and generate extreme hyperglycemia [48]. In summary, the β-cell mass parameters determine the number of fixed points, as well as the glucose level at the physiological and saddle points, when they exist.

5. Pathways into Diabetes

Defining diabetes as persistent hyperglycemia, there are three general pathways into diabetes in the βIG model: (1) move the physiological fixed point to a hyperglycemic level, (2) eliminate the physiological and saddle points, and (3) drive a trajectory across the separatrix. These pathways into diabetes will be referred to as regulated hyperglycemia, bifurcation, and dynamical hyperglycemia (or catch and pass), respectively.

5.1. Regulated Hyperglycemia

Defects in β-cell mass regulation can shift the physiological fixed point to a hyperglycemic level. Since steady state glucose levels are determined by β-cell mass dynamics, there are two possible means of shifting the physiological fixed point to a hyperglycemic level: (1) a defect in β-cell mass regulation, or (2) a loss of β-cell mass

regulation combined with a defect in glucose and/or insulin dynamics. Small defects in any of the β-cell mass parameters, for example a change in the response of replication and/or death rates to glucose, cause the physiological fixed point to shift to a hyperglycemic level (Figure 1-4A). The β-cell mass is responsive to changes in plasma glucose concentrations and has a basin of physiological attraction, but the physiologically regulated glucose level is now hyperglycemic. Bernard *et al.* (1999) showed that rats made hyperglycemic by STZ have β-cells capable of increasing replication and decreasing death rates in response to glucose infusion; yet in the absence of glucose infusion, the rats remained hyperglycemic [49].

A second way to shift the physiological fixed point to a hyperglycemic level is to couple a loss of β-cell mass regulation to an abnormality in glucose and/or insulin dynamics. Setting each of the β-cell mass parameters to zero results in a situation where replication and death rates are equal at all glucose levels, and as a result, the β-cell mass becomes non-responsive. In this situation, the βIG model reduces to the two-dimensional fast subsystem and defects in any of the glucose or insulin parameters can generate fasting hyperglycemia. This pathway fits intuitively with the human autopsy data of Kloppel *et al.* [2]. Pancreas from obese non-diabetic subjects had a larger than normal β-cell mass while obese people with diabetes had normal β-cell mass levels. In the latter subjects, a coupling of insulin resistance, due to obesity, to a non-adaptive β-cell mass may have been the cause of their diabetes. Figure 1-4B shows the effects of reduced insulin sensitivity on the fasting blood glucose and insulin levels assuming a non-adaptive β-cell mass.

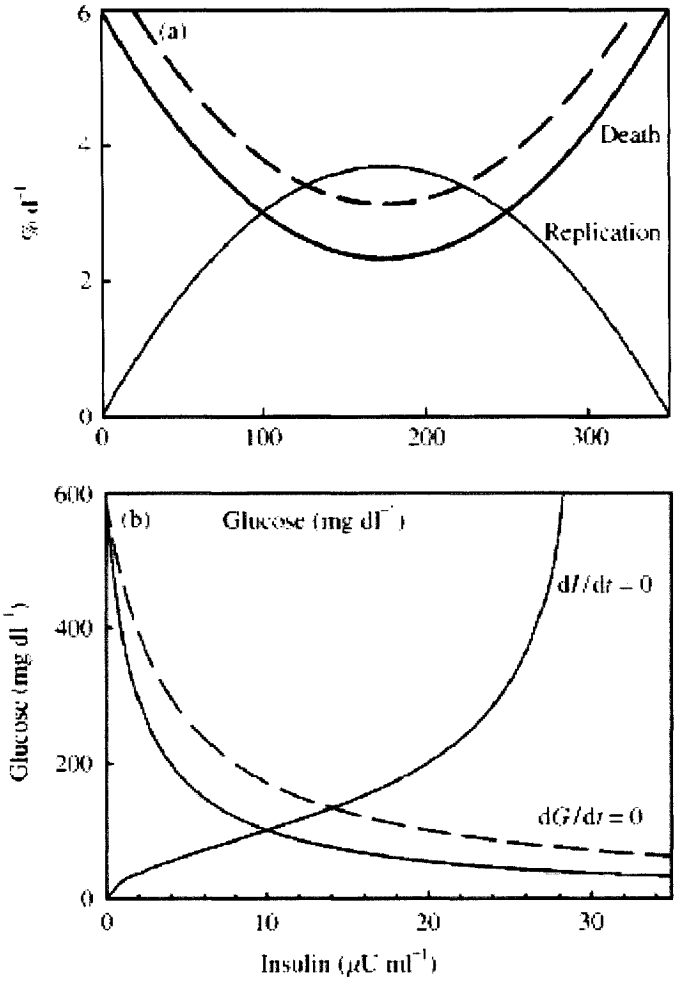

Figure 1- 4 Regulated hyperglycemia. There are two means of generating regulated hyperglycemia: a mild β-cell mass defect, or a loss of β-cell mass regulation (dβ/dt = 0 for all glucose levels) coupled to a defect in glucose and/or insulin dynamics. (A) shows a small upward shift of the death rate curve (dashed line). This shift moves the physiological fixed point to a higher glucose level and shrinks the basin of physiological attraction. (B) shows a reduction in insulin sensitivity. This changes the curvature of the glucose nullcline (dashed line) and shifts the steady state of the fast subsystem to a hyperglycemic level.

5.2. Bifurcation

The bifurcation pathway consists of any combination of parameter changes that result in the elimination of the physiological and saddle fixed points. Large defects in β-cell mass dynamics cause a saddle-node bifurcation, resulting in the single globally attractive pathological fixed point at zero β-cell mass. This occurs when death rates exceed replication rates at all glucose levels (Figure 1-5). A bifurcation diagram is shown in

Figure 1-6. The dashed line represents the saddle point while the solid lines represent the two stable fixed points. As r_1 decreases to r_{1c}, the saddle and physiological points move closer together, eventually colliding at a saddle-node bifurcation. At values of $r_1 < r_{1c}$, there only exists the pathological fixed point. At $r_1 = 0.0015$ dl/mg a transcritical bifurcation occurs involving the saddle point and the pathological fixed point. For values of $r_1 > 0.0015$ dl/mg the β-cell mass at the saddle point is negative and thus irrelevant physiologically. Similar bifurcation diagrams can be generated for r_2 and d_0. The bifurcation pathway outlined above resembles type 1 diabetes mellitus. An autoimmune attack on the pancreatic β-cells increases death rates above replication for all glucose levels, and the β-cell mass falls towards zero [48].

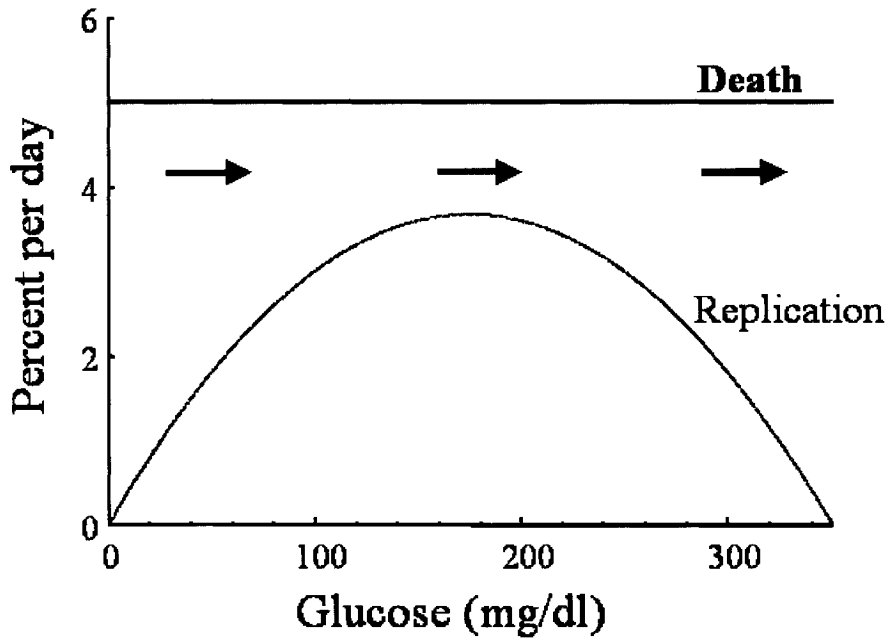

Figure 1- 5 Bifurcation. Large changes in the replication and/or death curves results in a situation where the death rate exceeds the replication rate for all glucose levels. Here the physiological and saddle points are eliminated. As the β-cell mass falls, insulin levels fall, and glucose levels are driven up. Under these conditions the system has a single globally attractive pathological fixed point at zero β-cell mass.

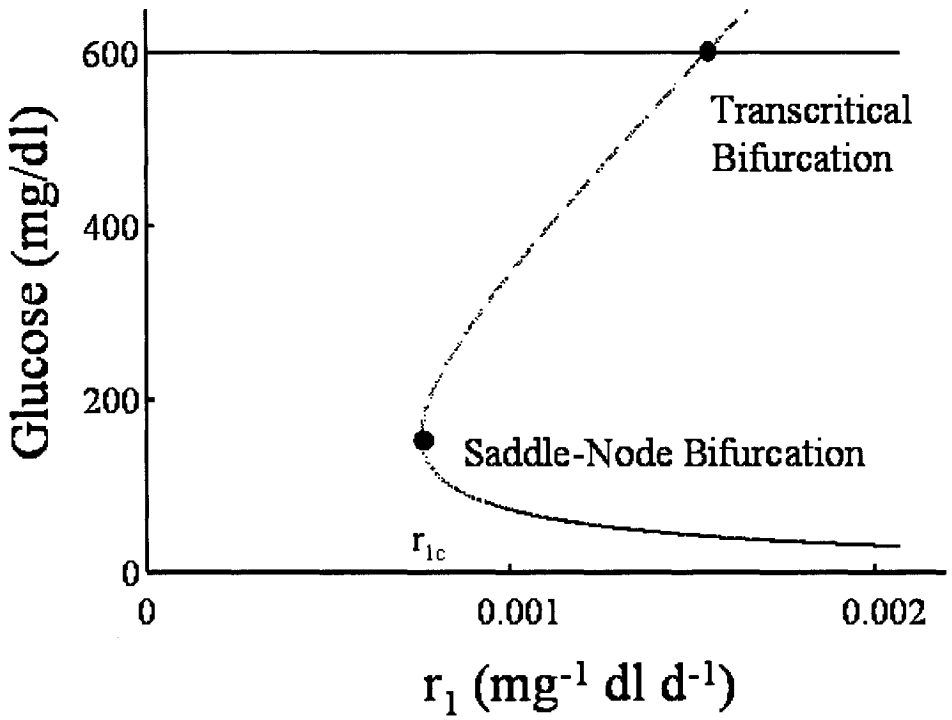

Figure 1- 6 Bifurcation diagram. The glucose levels of the stable fixed points (solid lines) and the saddle point (dashed line) are plotted as a function of the parameter r_1. Keeping all other parameters fixed constant, a saddle-node bifurcation occurs at the critical value of $r_{1C} = 0.00076$. The βIG model has a single pathological fixed point for $r_1 < r_{1C}$, and three fixed points (a physiological stable spiral, a saddle, and a pathological stable node) for $r_1 > r_{1C}$. A transcritical bifurcation occurs at $r_1 = 0.0015$. Model behavior to the right of this point is not physiologically interesting since fixed points with a glucose level greater than 600 mg/dl have negative β-cell mass.

5.3. Dynamic Hyperglycemia

Dynamical hyperglycemia, which we also refer to as the "catch and pass" pathway, is a race between parameter changes which increase the β-cell mass that is required to maintain normal glucose levels and adaptation of the actual β-cell mass. Defects in glucose and/or insulin dynamics do not affect the glucose level at the physiological fixed point, but do increase the β-cell mass required to maintain that glucose level. Thus, a step change in one of these parameters will generate a transient state in which there is insufficient β-cell mass to maintain normal blood glucose levels. Hyperglycemia persists

while the β-cell mass adapts. A time dependent shift in one or more of these parameters results in a race between a progressive defect, which drives glucose levels up, and β-cell mass adaptation, which drives glucose levels down.

Figure 1-7 (solid lines) shows the β-cell mass at the physiological and saddle points as a function of insulin sensitivity. In zone I, above the curve of physiological fixed points, glucose levels are below 100 mg/dl and the β-cell mass is decreasing. In Zone II, between the curves of physiological and saddle points, glucose levels range from 100 mg/dl to 250 mg/dl and the β-cell mass is increasing. All points in Zones I and II constitute the basin of physiological attraction. In Zone III, below the curve of saddle points, in region III, glucose levels are above 250 mg/dl and the β-cell mass is decreasing. All points below the curve of saddle points constitute the basin of pathological attraction. Notice that at large values of S_I, large changes in insulin sensitivity can be offset by relatively small changes in β-cell mass. However, at small values of insulin sensitivity, any change in S_I must be met with a dramatic change in β-cell mass.

We now investigate the adaptation of the β-cell mass in response to a continuous change in S_I, modeled by

$$dS_I/dt = -cS_I \tag{13}$$

where c is the rate constant (d^{-1}). Two trajectories are superimposed on Figure 1-7: normal adaptation (c = 0.01, dashed curve) and the catch and pass pathway (c = 0.05, dotted curve). Note that both trajectories start at the physiological steady state of G = 100 mg/dl for $S_I = 0.72\ \mu U^{-1}ml\ day^{-1}$. In the first case (c = 0.01), the actual β-cell mass adapts at roughly the same rate as the increase in β-cell mass of the physiological fixed point. Thus, the trajectory remains in Zone II, close to the curve of physiological fixed points and mild hyperglycemia is maintained throughout the simulation. In the case of the catch and pass pathway, however, the decrease in S_I is too fast. The actual β-cell mass increases, but not fast enough, so that the trajectory moves away from the curve of physiological fixed points and towards the curve of saddle points. Eventually, the

trajectory crosses the curve of saddle points into Zone III, the basin of pathological attraction. Once there, the β-cell mass along the trajectory begins to decrease, accelerating the rise in blood glucose levels. Note that the catch and pass pathway also can be realized with a slowly decreasing S_I coupled with a defect in β-cell mass dynamics. The catch and pass pathway is similar to the adaptation followed by a fall off of blood insulin levels observed in the Zucker Diabetic Fatty (ZDF) rat [50] and in cross-sectional human data [18]. However, it is likely that the insulin dynamics observed *in vivo* also are influenced by adaptation and/or failure of β-cell secretion rates as well as changes in insulin clearance rates that are not accounted for in our model.

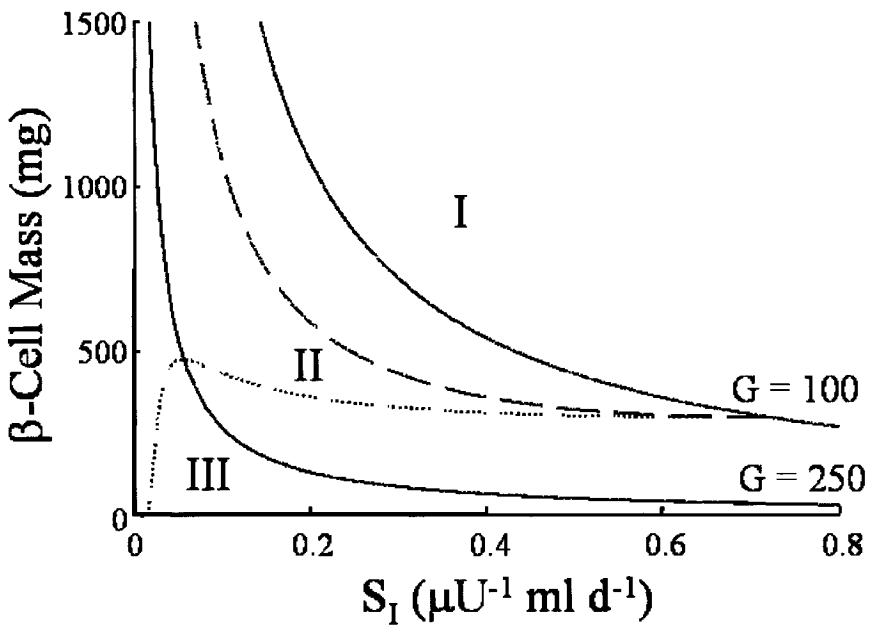

Figure 1- 7 Dynamic hyperglycemia. The solid lines represent the β-cell mass of the physiological (upper curve) and saddle fixed points (lower curve) as a function of insulin sensitivity, S_I. The derivative dβ/dt is negative in Zone I (above the line of physiological fixed points), and III (below the line of saddle points), but is positive in Zone II (between the line of physiological points and the line of saddle points). If S_I is reduced slowly (c = 0.01), then the β-cell mass can adapt and maintain mild hyperglycemia (dashed line). However, if S_I is reduced quickly (c = 0.05), then the β-cell mass adapts for some period of time, but glucose levels are eventually driven above 250 mg/dl where the β-cell mass begins to decrease (dotted line).

6. Discussion

In this paper we present a novel model of coupled β-cell mass, insulin, and glucose dynamics which we employ to investigate the normal behavior of the glucose regulatory system and the pathways into diabetes. The model predicts a physiological steady state with a large basin of attraction. Blood glucose levels greater than 250 mg/dl cause β-cell death rates to exceed replication rates, driving the system towards a pathological steady state. The behavior of the model is consistent with the observed behavior of the glucose regulatory system in response to changes in blood glucose levels [6, 22, 45], β-cell mass [51], insulin sensitivity [15], and cellular insulin secretion rates [47]. Furthermore, the model predicts three characteristic pathways into diabetes: regulated hyperglycemia, bifurcation, and dynamic hyperglycemia (catch and pass). Although it is difficult to quantify the extent to which these pathways correspond to the behavior of the real system, the regulated hyperglycemia pathway seems consistent with STZ-induced diabetes [49], and the bifurcation pathway has strong parallels with the pathogenesis of type 1 diabetes mellitus [48]. The catch and pass pathway provides a novel theoretical explanation for observed insulin dynamics in longitudinal studies of the ZDF rat [50] and in cross-sectional human studies [18].

The idea that "glucose toxicity" or some other factor leads to β-cell "exhaustion" has been around for many years [19]. We suggest that nonlinear β-cell mass dynamics can explain the adaptation of plasma insulin levels to insulin resistance whereas the failure of this adaptation is followed by, hyperglycemia, and reduced plasma insulin levels. The βIG model predicts that blood glucose levels are determined by the rate of change of insulin demand, compared to the rate of adaptation of the β-cell mass. Moderate levels of hyperglycemia (below the saddle point) leads to a negative feedback and adaptation of the β-cell mass. At higher plasma glucose levels (above the saddle point), adaptation fails, and positive feedback leads to a decrease in the β-cell mass, further increasing the glucose level. The βIG model provides (1) a framework for developing experimental protocols to test specific hypotheses regarding β-cell exhaustion

and the pathogenesis of type 2 diabetes, and (2) a tool for investigating the behavior of β-cell mass, plasma insulin, and plasma glucose, in response to therapeutic interventions.

The equation describing β-cell mass dynamics is based on a limited number of *in vitro* experiments, which support the hypothesis that β-cell replication and apoptosis rates are nonlinear with respect to blood glucose levels. Other endogenous signals may be important in control of β-cell mass dynamics, but it remains to be determined whether these signals act independently or whether they modify the relationship between plasma glucose and β-cell turnover. Clearly, additional experimental work is needed to refine the model further, but the fundamental relationship between β-cell mass dynamics and the plasma glucose level will probably remain.

The model does not incorporate all known physiological effects, for example, the effects of hyperglycemia on neogenesis [49], insulin sensitivity [21], insulin secretion rates [52], and β-cell heterogeneity [53]. Although these effects have been demonstrated experimentally, they have not been quantified, and their incorporation into the βIG model would require speculation about the rate and the magnitude of these events. Moreover, preliminary analysis and simulations of some speculative models (data not shown) indicate that these physiological responses do not affect the qualitative behavior of the βIG model or the predicted pathways into diabetes.

Experimental studies have indicated a potentially important role of lipid metabolism in the regulation of blood glucose levels and the pathogenesis of type 2 diabetes [54]. Insulin dynamics are an important component of lipid metabolism [55], and plasma fatty acid levels have been linked to insulin resistance [56], insulin secretion defects [57], and abnormal β-cell mass dynamics [58]. The incorporation of lipid dynamics into the βIG model may affect the present pathways into diabetes or reveal novel pathways not evident with the present form of the βIG model.

Finally, the βIG model may provide the theoretical basis for an indirect measurement of β-cell mass *in vivo*. Present techniques for measuring β-cell mass are time consuming and require removal of the pancreas. However, $\sigma\beta$, from eqn (12), can be calculated from *in vivo* glucose clamp data, and σ can be measured *in vitro*. Thus, utilizing the *in vitro* population mean value for σ, or developing a means of measuring σ *in vivo*, would permit the estimation of β-cell mass *in vivo*. This would eliminate the need for removing the pancreas, allow for longitudinal studies of β-cell mass dynamics, and would facilitate the study of β-cell mass dynamics in humans.

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Appendix

Define

$$G_0 = 100 \text{ mg/dl}, I_0 = 10\mu\text{U/ml}, \beta_0 = 300 \text{ mg},$$

and let

$$G = G_0\bar{G}, I = I_0\bar{I}, \beta = \beta_0\bar{\beta}, t = \tau\bar{t}.$$

Substituting these into (8) yields

$$(G_0/\tau)(d\bar{G}/d\bar{t}) = R_0 - (E_{G0}G_0 + S_IG_0I_0\bar{I})\bar{G},$$
$$(G_0/\tau R_0)(d\bar{G}/d\bar{t}) = 1 - (E_{G0}G_0/R_0 + (S_IG_0I_0/R_0)\bar{I})\bar{G}.$$

Defining

$$\tau = G_0/R_0, E_{G0}^* = E_{G0}G_0/R_0, \text{ and } S_I^* = S_IG_0I_0/R_0,$$

gives the dimensionless glucose equation

$$d\bar{G}/d\bar{t} = 1 - (E_{G0}^* + S_I^*\bar{I})\bar{G}. \tag{18}$$

Equation (12) becomes

$$(I_0/\tau)(d\bar{I}/d\bar{t}) = \beta_0\bar{\beta}\sigma G_0^2\bar{G}^2/(\alpha + G_0^2\bar{G}^2) - kI_0\bar{I},$$

or

$$d\bar{I}/d\bar{t} = (\tau\beta_0\sigma/I_0)(\bar{\beta}\bar{G}^2/(\alpha/G_0^2 + \bar{G}^2)) - k\tau\bar{I},$$

and defining

$$\sigma^* = \tau\beta_0\sigma/I_0, \alpha^* = \alpha/G_0^2, \text{and } k^* = k\tau,$$

we arrive at the dimensionless insulin equation

$$dI^{\sim}/dt^{\sim} = \sigma^{*}\beta^{\sim}G^{\sim 2}/(\alpha^{*} + G^{\sim 2})) - k^{*}I^{\sim}. \quad (19)$$

Equation (16) becomes

$$(\beta_0/\tau)(d\beta^{\sim}/dt^{\sim}) = (-d_0 + r_1G_0G^{\sim} - r_2G_0^2G^{\sim 2})\beta_0\beta^{\sim},$$

or

$$d\beta^{\sim}/dt^{\sim} = (-d_0\tau + r_1\tau G_0G^{\sim} - r_2\tau G_0^2G^{\sim 2})\beta^{\sim},$$

and defining

$$d_0^{*} = d_0\tau,\ r_1^{*} = r_1\tau G_0, \text{ and } r_2^{*} = r_2\tau G_0^2,$$

gives us the dimensionless β-cell mass equation

$$d\beta^{\sim}/dt^{\sim} = (-d_0^{*} + r_1^{*}G^{\sim} - r_2^{*}G^{\sim 2})\beta^{\sim}. \quad (20)$$

Substituting the normal parameter values (cf. Table 1) into (18), (19), and (20) yields

$$dG^{\sim}/dt^{\sim} = 1 - (0.16 + 0.83I^{\sim})G^{\sim},$$
$$dI^{\sim}/dt^{\sim} = 100(3\beta^{\sim}G^{\sim 2}/(2 + G^{\sim 2}) - 0.5I^{\sim}),$$
$$d\beta^{\sim}/dt^{\sim} = 0.06(-1 + 0.162G^{\sim} - 0.046G^{\sim 2})\beta^{\sim}.$$

Noting the smallness of the factor 0.06 in the equation for $\beta^{\sim}$, we conclude that the glucose and insulin dynamics are much faster than the β-cell mass dynamics.

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CHAPTER 2: APPLICATION OF THE βIG MODEL

Metabolic Adaptations to Chronic Glucose Infusion

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Abstract

Aims/hypothesis. Several studies have employed the chronic glucose infusion protocol to quantify the metabolic adaptations associated with a prolonged glucose challenge. However, the limited number of indices and time points reported by these studies has generated an incomplete picture of this process. Here we generate an integrative and dynamical picture of the physiological adaptations that occur during chronic glucose infusion.

Methods: Sprague Dawley rats were infused with 50% dextrose or saline (2 ml/hr) for 0-6 days. Glucose, insulin and NEFA dynamics were determined from daily blood samples. Subsets of animals were sacrificed daily for histological determination of β-cell mass, size and replication rates. The βIG model was used to estimate insulin sensitivity, β-cell function, and net-neogenesis from this data.

Results. Glucose infused rats displayed transient hyperglycaemia, persistent hyperinsulinemia and unchanged NEFA levels. Insulin sensitivity decreased by ~80 % during the first day of glucose infusion, but returned to pre-infusion levels by day 3. β-cell function was 4-6 fold higher than control throughout the experiment. β-cell mass doubled over the 6 days of glucose infusion due to an initial wave of neogenesis, followed by hypertrophy and hyperplasia, then finally a second wave of β-cell neogenesis coupled to continued hyperplasia.

Conclusions/interpretation. Contrary to perfused pancreas and *in vitro* data, we found chronic glucose infusion to elevate β-cell function. Also, the prediction of a second wave of β-cell neogenesis, coupled with our previous report of "focal areas" on day 3, suggests the existence of delayed acinar-to-islet transdifferentiation.

Introduction

Since its first application in the 1980's [1], chronic high dose glucose infusion has been used to quantify the effects of increased glucose supply on insulin sensitivity [2, 3, 4], β-cell function [5, 6, 7, 8, 9], β-cell mass [6, 10, 11], and indices of β–cell replication [10, 11], death [10], and neogenesis [10]. However, the limited number of variables and time points presented in these studies has lead to an incomplete understanding of this process. For example, hyperglycaemia has long been known to induce insulin resistance in muscle. However, recent studies have shown prolonged glucose infusion to lead to augmented insulin action in adipose tissue [3, 4]. The time course and sustainability of this adaptation remains unclear. Also, several *in vitro/in situ* studies have suggested that β–cell function is impaired by chronic glucose infusion while *in vivo* acute insulin response studies have shown the opposite [5, 6, 7, 8]. Finally, while several studies have reported morphological evidence of neogenesis, apoptosis, or transdifferentiation, it is unclear if changes in these markers reflect significant contributions of these processes to the observed β–cell mass dynamics [6, 10, 11]. The tendency for existing studies to focus on only one of insulin resistance, or β–cell function, or β–cell mass is based partly on the time, expense, and expertise associated with existing *in vivo* methodologies such as the hyperinsulinemic clamp, perfused pancreas and quantitative histology. Similar arguments explain the limited number of time points typically reported in these studies, while the inability to quantify the relative contributions of death and neogenesis to overall β–cell mass dynamics lies in an incomplete understanding of how long it takes a labelled cell to go from one stage to another (i.e. alive to engulfed or duct/acinar to β–cell). Together this suggests that novel *in vivo* methodology or data analysis techniques need to be developed before a truly integrative and dynamical analysis of this process can occur.

While advancements in longitudinal *in vivo* imaging techniques hold great potential for indirect assessment of cellular population sizes or gene transcription rates, mathematical modelling has already demonstrated an ability to generate indirect assessment of metabolic processes from easily obtained experimental data. Examples of this include the

HOMA [12] and QUICKIE [13] models which have been widely used to estimate insulin sensitivity and/or β–cell function from fasting glucose and insulin levels. In addition to indirect assessment of metabolic indices, mathematical modelling has been used to generate indices which can not be determined histologically, such as rates of β–cell death/neogenesis *in vivo* [14]. Recently, we developed a mathematical model of coupled β–cell mass, insulin, and glucose (βIG) dynamics. While initially designed as a bifurcation and simulation analysis tool, we found that the βIG model could also be used as a tool for generating indirect estimates of insulin sensitivity, β–cell function, and net β–cell neogenesis [15]. These indices have been shown to correlate strongly with Minimal model and HOMA derived indices in a large human data set covering the entire range of glucose tolerance [16]. However, unlike the HOMA, QUICKIE, or Minimal models, the βIG model can be easily re-fitted with species specific parameter values and applied to *in vivo* data.

Here we use an experimental and theoretical methodology to generate an integrative and dynamic analysis of the metabolic adaptations to chronic glucose infusion. Daily estimates of glucose, insulin, NEFA levels as well as β–cell mass, size, and replication rates are determined experimentally in Sprague Dawley rats infused with 50% dextrose or saline for 0-6 days. The βIG model is then used to generate daily estimates of insulin sensitivity, β–cell function, and net neogenesis from this experimental data. We found that insulin sensitivity displays a transient reduction (~80% reduced on day 1 and 2) then returns to normal for the duration of the experiment. Similar to other studies that used *in vivo* methods for determining β–cell function, we found individual insulin secretory capacity to be elevated during the first 4 days of glucose infusion. Finally, analysis of our β–cell mass data suggests that the linear expansion of the β–cell mass observed during this study is composed of three phases: neogenesis (day 1), hypertrophy and hyperplasia (days 2-3), then neogenesis and hyperplasia (days 4-6). The prediction of a second wave of β–cell neogenesis, combined with our previous discovery of "focal areas" on day 3 of infusion, supports the possibility of late acinar to islet transdifferentiation in this experimental model.

Methods

Animals. The infusion protocol was similar to that of Bonner-Weir *et al.* [11]. Male Sprague-Dawley rats (~230 g) were housed in plastic shoebox cages at 22°C on 12L:12D photoperiod and given free access to food and water. Under anesthesia (sodium pentobarbital, 35 mg/kg i.p.) each rat was fitted with an indwelling jugular catheter (Intramedic PE50), which was exteriorized at the nape of the neck and connected by flexible tether to a swivel system. The tethers were attached to lightweight stretch cotton vests that were custom-made to fit the rats, allowing for free movement within the cage. All procedures adhered to the standards of the Canadian Council on Animal Care and were approved by the Health Sciences Animal Welfare Committee at the University of Alberta and the Animal Care Committee of Simon Fraser University. Histological analysis of pancreatic tissue from these animals has been previously published [34].

During recovery from surgery, catheter patency was maintained by a slow infusion of 0.9% saline. After 3-5 days, rats were either euthanized (Day 0, n = 10) or infused (2 ml/h) with 0.45% saline (n = 40) or 50% dextrose in 0.45% saline (n = 65) for 1, 2, 3, 4, 5 or 6 days. Blood samples from tail snipping were taken daily. Animals were allowed free access to regular chow and water during the infusion period. Six hours before sacrifice on the final infusion day, each rat was injected with 100 mg/kg 5-bromo-2'deoxyuridine (BrdU, Amersham Canada Ltd., Oakville ON), a thymidine analogue incorporated into newly synthesized DNA. At the time of killing, rats were anesthetized (sodium pentobarbital 35 mg/kg i.p.) and a blood sample was taken by cardiac puncture. The pancreas was rapidly excised and dissected free from surrounding connective and adipose tissue. Each pancreas was cut into 3 pieces, which were blotted, weighed and placed in separate cassettes. The tissue was fixed overnight in Bouin's solution, then washed for 8h in cold running water and stored in 10% buffered formalin until embedded in paraffin.

Plasma assays. Blood samples were kept on ice until centrifuged and the plasma was stored at -20 °C until assayed. Plasma glucose was measured by the glucose oxidase

method (Trinder, Sigma Diagnostics, St. Louis, MO) on duplicate 5 µl samples using a microplate reader. Plasma NEFA were determined using an enzymatic colorimetric method (ACS-ACOD, Wako Chemicals USA, Inc., Richmond, VA) on duplicate 4 µl samples using the microplate reader. Plasma insulin was assayed by radioimmunoassay using a specific anti-rat insulin antibody and rat insulin standards (Linco Research, Inc., St. Louis, MO). The detection limit was 0.1 ng/ml and intra- and inter assay coefficients of variation were < 10%.

Immunocytochemistry. Sections (4-5 µm thick) from each of the 3 pieces of pancreas were de-waxed in xylene. Endogenous peroxidase activity was blocked with 0.3% solution of hydrogen peroxide. The sections were then washed with phosphate buffered saline (PBS) and incubated with 10% lamb serum in PBS for 30 minutes at room temperature. Slides for determination of β–cell mass and size were stained with a cocktail of antibodies to glucagon, somatostatin, and pancreatic polypeptide (rabbit anti-human, 1:2000, Dako Diagnostics Canada Inc., Mississauga ON) for identification of islet non-β–cells (alpha, delta, PP cells). Slides for determination of β–cell replication were double stained with the cocktail and anti-BrdU antibody (mouse monoclonal anti-BrdU, 1:100, Amersham Canada Ltd., Oakville ON). Slides were incubated with primary antibodies overnight at 4 °C before being washed in PBS and incubated with the secondary antibodies (1:500, biotinylated goat anti-guinea pig IgG, biotinylated goat anti-mouse IgG or biotinylated goat anti-rabbit IgG, Dako) for 1 hour at room temperature. Sections were then washed in PBS, incubated with avidin-biotin complexed with horseradish peroxidase (1:1000, Vectastain Elite ABC Kit, Vector Laboratories Canada Inc., Burlington ON) for 1 hour and then developed using 3,3'-diaminobenzidine tetrahydrochoride (DAB, Sigma-Aldrich Canada Ltd., Oakville ON). All slides were counterstained with hematoxylin (Sigma-Aldrich).

β–cell mass. β–cell mass was determined from cocktail-stained sections using an image analysis system. This system consisted of an Olympus light microscope (Model BX40, Carsen Group Inc.) attached to Sony colour video camera (Model DXC-950, Sony Co.,

Japan) and Northern Eclipse software (Empix Imaging Inc., Mississauga ON). Each section was stepped through in 1.5 x 1.5 mm stage increments (at 400X). For each field the areas corresponding to pancreas (exocrine and endocrine), non-pancreas (fat, space, connective tissue) and islet (β and non-β–cells) were determined. Total islet area was determined by hand-tracing each islet perimeter identified by the mantle of non-β–cells and the morphology of the tissue. Non-β–cell area was determined by distinguishing DAB stained tissue within the hand traced region of interest using the threshold function of the image analysis software. β–cell area was calculated as the difference between total islet area and non-β–cell area. Islet area relative to total tissue area was determined by using the threshold function to quantify total tissue area stained with hematoxylin and DAB in each field. For fields containing adipose tissue, the area of "white space" previously occupied by lipid was determined separately and added to the total tissue area. β–cell mass was calculated as the relative area of tissue occupied by β–cells multiplied by the wet weight of the segment of pancreas. Data from individual portions of each pancreas were summed.

β–cell size. Measurements of individual β–cell area were made in islets having ≥ 10 β–cells, since significantly greater measurement error was associated with smaller islets (data not shown). Cocktail-stained sections were scanned and every islet ≥ 10 β–cells was assessed for β–cell size. β–cell area was determined as described above by hand tracing the islet perimeter and thresholding for non-β–cells stained by cocktail. The number of nuclei in the β–cell area was counted and the average single β–cell cross sectional area was calculated as the total β–cell area divided by the number of nuclei. This method has been shown to overestimate average β–cell size in comparison to electron microscope determinations, but has been considered acceptable for comparison of treatment effects [10, 17]. β–cell volume was estimated from the cross sectional area based on the calculated radius (area = πr^2) and the volume of a sphere (volume = $4/3\pi r^3$). The average β–cell size (μg) is represented by the symbol β_s.

β–cell replication. Sections double stained with cocktail and anti-BrdU antibody were used to determine β–cell replication. BrdU incorporation was determined by systematic sampling all of the β–cells identified with cocktail staining in each section, using a light microscope under high magnification (x1000). β–cells incorporating BrdU (positive) had blue/black nuclei.

βIG model estimates of insulin sensitivity, β–cell function and net-neogenesis. A schematic of the βIG model feedback loops can be found in Figure 2-1. Details of the βIG model data analysis can be found in the appendix.

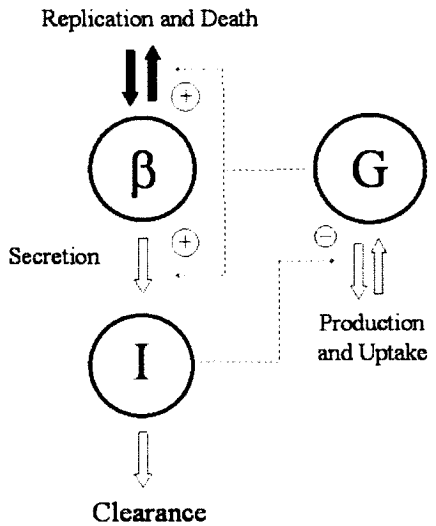

Figure 2- 1 A mathematical model of coupled β–cell mass, insulin, and glucose (βIG) dynamics. Here glucose regulates blood insulin levels by increasing insulin secretion rates on a fast time scale, and by augmenting β–cell replication and death rates on a slower time scale.

Statistics. Data are presented as mean ± standard error unless otherwise noted. For the measured variables (glucose, insulin, β–cell mass, BrdU incorporation, β–cell cross-sectional area), the effect of treatment (saline vs. glucose infusion) and time were determined by analysis of variance with post hoc Tukey comparisons. Unless otherwise stated, $p < 0.05$ was considered significant. All statistical calculations were performed using SAS Version 7.0 (The SAS Institute, Cary, NC).

Results

Body and pancreas weight. Body and pancreatic weight are displayed in Table 1. Except for day 4 of infusion, body weight did not differ between groups. While there was a general tendency for the animals to gain weight with increasing length of infusion of either type, this was only significant on day 5 of infusion and may be due in part to differences between groups prior to surgery (data not shown). Pancreas mass was ~35% lower in glucose-infused rats on all infusion days except Day 5 (Table 1).

Table 2- 1 Body mass, and pancreas mass of saline- and glucose-infused rats.

Variable	Infusion Day	Saline-infused	N	Glucose-Infused	N
Body mass					
(g)	0	235.6 ± 4.4	10		
	1	224.9 ± 4.1	7	228.9 ± 6.7	10
	2	236.8 ± 6.8	6	232.4 ± 5.9	10
	3	242.7 ± 9.7	8	251.5 ± 7.7	18
	4	246.1 ± 5.1	9	231.1 ± 3.0 #	13
	5	264.6 ± 6.0 *	6	274.1 ± 7.0 *	8
	6	262.3 ± 4.8	4	267.7 ± 9.9	5
Pancreas mass					
(mg)	0	1176.3 ± 128.7	10		
	1	916.9 ± 31.7	7	725.6 ± 33.4 *#	10
	2	993.8 ± 29.9	6	761.1 ± 43.7 *#	10
	3	1072.9 ± 33.4	8	804.8 ± 31.8 *#	18
	4	980.6 ± 41.2	9	701.0 ± 22.2 *	13
	5	1147.7 ± 79.0	6	900.3 ± 47.8 *#	8
	6	1128.5 ± 53.0	4	722.8 ± 66.6 *#	5

* indicates significant difference at $p < 0.05$ as compared to day 0 while # indicates a significant difference at $p < 0.05$ between saline- and glucose-infused animals.

Plasma data. Saline infusion led to a slight (~15%) reduction in plasma glucose levels but had no effect on plasma insulin concentrations (Figure 2-2). Non-esterified fatty acid (NEFA) levels tended to increase with time in saline infused animals. The glucose

infused animals displayed marked hyperglycaemia on day 1 and 2 of infusion. Glucose returned to pre-infusion levels by day 3 and remained low throughout the experiment. Insulin levels increased from 180 ± 12 to 2256 ± 306 pmol during the first day of infusion and decreased to 1476 ± 792 pmol by day 5 (Figure 2-2). NEFA levels rose on day 1 then returned to day 0 levels by day 2 and thus tended to be lower than levels observed in saline infused animals. Note that glucose and insulin dynamics were derived from tail vein blood sample while NEFA levels were determined from cardiac puncture blood samples that were taken at the time of sacrifice. Tail vein blood samples were not taken on the day of sacrifice, thus glucose and insulin data is not available on day 6 of infusion.

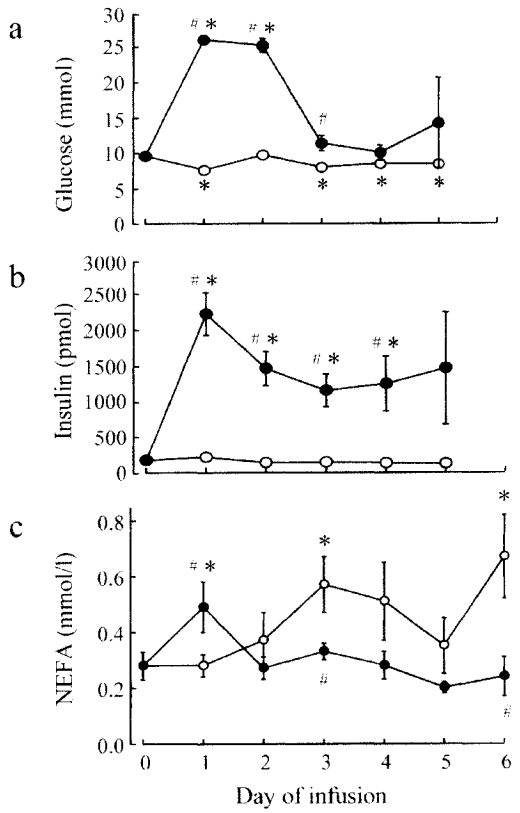

Figure 2- 2 Venous plasma glucose (a) insulin (b), and NEFA (c) levels in saline (O) and glucose (●) infused rats. # indicates significant difference between treatment groups while * indicates a difference from day 0, ($p < 0.05$).

Histological analysis of pancreas. There was no effect of saline infusion on the level of β–cell mass or individual β–cell size; however, saline infusion did have a small but significant negative effect on BrdU incorporation on day 3 and 5 of infusion. Glucose

infused animals, on the other hand, displayed an approximately linear two-fold increase in β–cell mass (5.1 ± 0.3 mg to 10.0 ± 0.9 mg). Replication rates, as assessed by BrdU incorporation, displayed a nonlinear time course decreasing on day 1, increasing until day 4, and then remained elevated on days 5 and 6. Average β–cell size increased by ~25% between days 1 and 3 of glucose infusion (Figure 2-3).

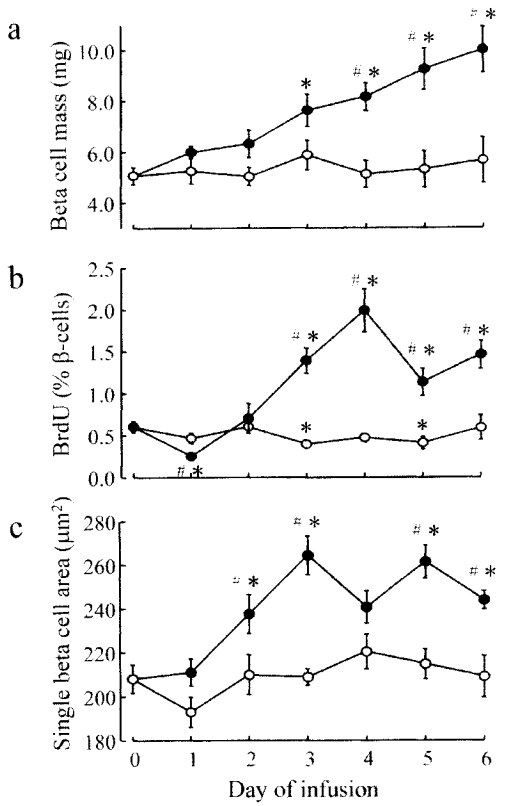

Figure 2- 3 β–cell mass (a), BrdU incorporation into β–cells (b), and β–cell area (c) in saline (○) and glucose (●) infused rats. # indicates significant difference between groups while * indicates significant difference from day 0, ($p < 0.05$).

βIG model analysis. Insulin sensitivity remained statistically unchanged in the saline group, but did display an increasing trend (Figure 2-4). This reflects the observation of modestly decreasing glucose levels during a period of sustained insulinemia. While the dynamics of insulin sensitivity are reasonably intuitive in the saline group, the presence of a chronic glucose infusion complicates the calculation. However, βIG model analysis

of this data suggests that glucose levels on day 1 and 2 of infusion are higher than can be accounted for by the chronic glucose infusion alone (considering the observed degree of insulinemia). This analysis suggests that whole body insulin sensitivity decreases by ~80% on day 1. While it is clear that insulin sensitivity increases between days 2 and 3 (as glucose levels drop dramatically while insulinemia and infusion rates remain unchanged) the magnitude of this change is non-intuitive. βIG model analysis of this data suggests that insulin sensitivity returns to, then remains at pre-infusion levels.

βIG model estimates of β–cell function for the two groups are presented in Figure 2-4. Here we are essentially asking the question: are the observed insulin levels higher or lower than one would expect for the prevailing glucose and β–cell mass levels. The stable insulin levels displayed by the saline infused animals are consistent with the relatively unchanged glucose and β–cell mass levels observed in these animals. Consequently βIG model analysis of this data suggests that β–cell function remains unchanged. Glucose infused animals, on the other hand, display hyperinsulinemia beyond what would be predicted by the observed glucose and β–cell mass dynamics alone. βIG model analysis of this data suggests a transient 3-4 fold increase in β–cell function in these animals (Figure 2-4).

The observation of constant β–cell mass in the presence of constant β–cell replication rates (~2% per day) in saline infused animals clearly suggests that these animals display a constant rate of net neogenesis (neogenesis – death = -2% per day). β–cell mass dynamics in the glucose infused animals proved to be more complex. We fit the β–cell mass, cell size, and BrdU data with simple equations (linear, sigmoid, and piecewise linear equation respectively) then used this curve fit data to estimate net-neogenesis. Our calculations suggest glucose infused rats display two waves of net-neogenesis interrupted by a wave of net-β–cell death (Figure 2-4). Although we can not calculate independent estimates of neogenesis and β–cell death rate, this data does suggest that each of the two waves of neogenesis must be no smaller than 4% per day (as death rates are > 0% per

day). Similarly, the middle wave of β–cell death rates must incorporate death rates of at least 5% per day (as neogenesis can not be negative).

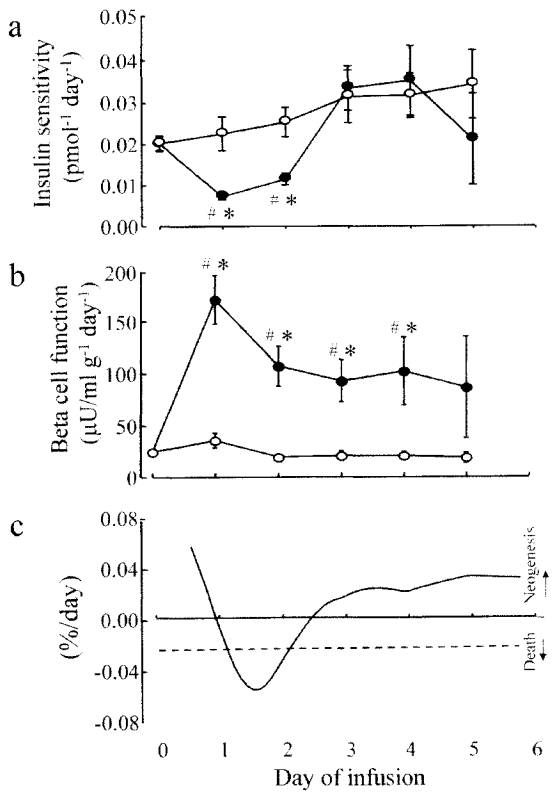

Figure 2- 4 βIG model derived estimates of insulin sensitivity (a) and β–cell function (b) in saline (O) and glucose (●) infused rats. βIG model estimates of net-neogenesis (c) are shown for saline (- - -) and glucose (—) infused rats. # indicates significant difference between groups while * indicates significant difference from day 0, ($p < 0.05$).

Discussion

Humans and animals with Type 2 diabetes display insulin resistance, insulin secretory defects, and insufficient (possibly reduced) β–cell mass [17, 18, 19, 20, 21]. Several authors have suggested that diabetes is characterized by insufficient β–cell adaptation to insulin resistance [15, 22, 23] followed by β–cell failure. While there is increasing evidence to suggest that glucose plays a critical role in β–cell failure [24, 25, 26, 27, 28] the role of glucose during the early phases of insufficient adaptation has been much debated. Some authors

have suggested that glucose is the primary regulator of β–cell mass and function *in vivo*, while others have noted β–cell adaptation in the absence of hyperglycaemia [5, 6, 8, 10, 15, 29, 30, 31, 32, 33]. To this end, several researchers have employed the chronic glucose infusion protocol to investigate the effects of chronic hyperglycaemia *per se* on β–cell mass, replication, death, neogenesis, β–cell function, and peripheral insulin action [1, 2, 3, 5, 6, 7, 8, 10, 11, 26]. Due to limitations of existing experimental techniques, these studies have each reported only a limited number of variables at a few points in time.

In this study, we utilized quantitative morphology and mathematical modelling to estimate the full dynamical response of each of these physiological variables during a chronic glucose infusion. Our findings were consistent with previous reports of transient insulin resistance and elevated β–cell function *in vivo* [3, 7]. We were also able to confirm the contributions of neogenesis, hypertrophy and hyperplasia in early expansion of the β–cell mass in this protocol. Novel findings from this study include a prediction of increased β–cell death during day 2 of infusion and the existence of a second wave of β–cell neogenesis that occurs during the second half of the study. This predicted second wave of neogenesis is consistent with our previous finding of "focal" areas on day 3 of chronic glucose infusion [34].

The predicted dynamics of whole body insulin sensitivity reported here are consistent with previous studies which have addressed this issue. Laybutt *et al.* found whole body insulin sensitivity was reduced on day 1 of glucose infusion, but returned to normal by day 4 [3]. The initial insulin resistance has been shown to be based largely in muscle and likely occurs as a direct result of the prevailing hyperglycemia [2, 3, 35]. Later increases in insulin sensitivity are due largely to increased glucose uptake by adipose tissue [3, 4]. The mechanisms regulating this adaptation are not well understood. The similarity of our model predictions to data from the literature, coupled to the previous observation of a strong correlation between βIG model and Minimal model estimates of insulin sensitivity [16], suggest that the dynamics of insulin sensitivity reported here are accurate. Thus while hyperglycaemia and hyperinsulinemia have long been shown to cause insulin resistance

[35, 36], the transient nature of this insulin resistance suggests that over the long term insulin sensitivity may be regulated by adipose tissue physiology.

Here, as in other studies, chronic glucose infusion led to persistent hyperinsulinemia despite transient hyperglycaemia. However, the literature describing the contributions of β–cell function to this hyperinsulinemia is mixed. Our findings are consistent with existing studies that have utilized *in vivo* methodologies to quantify β–cell function, but are in contrast with studies that have utilized *in situ* or *in vitro* methodologies to estimate glucose induced insulin secretion [5, 6, 7, 8, 37, 38]. This point is well illustrated by Laury *et al.* who performed both *in vivo* and *in situ* measurements of β–cell function following 4 days of glucose infusion and found insulin secretion to be elevated *in vivo* but reduced *in situ* [38]. Discrepancy between *in vitro* and *in vivo* indices of β–cell function may be related to changes in β–cell function during the isolation process or to some other blood born secretogogue.

We observed expansion of the β–cell mass at a rate that is similar to some, but slower than other chronic glucose infusion studies [6, 10, 11, 37]. Our β–cell replication and hypertrophy data are consistent with the previous reports [10, 11, 37]. Also the early wave of β–cell neogenesis predicted here is consistent with the previous finding of neogenesis at 24 but not 48 hr [10]. Our prediction of a wave of β–cell death during the second day of infusion is consistent with previous findings of hyperglycemia induced apoptosis, but inconsistent with the finding of Bernard *et al.* who reported a reduced number of TUNEL positive β–cells on each of days 1 and 2 of glucose infusion [10, 39]. First, it should be noted that both our and the Bernard *et al.* study reported increased replication, increased cell size, and relatively constant β–cell mass during the second day of infusion; data that intuitively suggests increased β–cell death during this time period. Second, our predicted wave of apoptosis was highly transient and peaked at 36 hours while Bernard *et al.* quantified TUNEL positive β–cells at 24 and 48 hours making it possible that in their study this brief wave was not detected. It is also important to remember that these two methods provide different indices of cell death which are dependent on different potential

sources of error. In the case of the model, errors or biases in β–cell mass and replication will affect the estimate of β–cell death, whereas use of the TUNEL method as an index of the *rate* of β–cell death requires the assumption that clearance of the apoptotic cells is not affected by the treatment [40]. Finally, this discrepancy may simply reflect differences in animal models (adult Wistar rat vs. young Sprague Dawley). Further investigation will be necessary if this difference is to be resolved.

Finally, our model based analysis of β–cell mass dynamics predicted a second wave of β–cell neogenesis between days 3 and 6 of glucose infusion. This is consistent with our previous finding of "focal areas" on day 3 of glucose infusion 34. These areas consisted of de-differentiated acinar tissue full of BrdU positive cells and small amounts of insulin staining similar to those previously reported by Bonner-Weir *et al* 41 following a partial pancreatectomy. Together, this data suggests a potentially important contribution of acinar-to-islet transdifferentiation to the β–cell mass adaptation that occurs during chronic glucose infusion.

Overall, the βIG model predictions presented here are largely consistent with the existing literature. Also, βIG model estimates of insulin sensitivity and β–cell function have been shown to correlate strongly with HOMA, QUICKI, and Minimal Model derived estimates of these parameters while our model based approach to estimating net-neogenesis has previously been used to predict the existence of a neonatal wave of β–cell apoptosis [16, 42]. Taken together these data suggest that the βIG model may provide a practical means of estimating the dynamics of insulin sensitivity, β–cell function, and net-neogenesis in animal models.

In summary, by employing a combined mathematical and experimental approach, we have been able to determine the most complete analysis of the metabolic adaptation to chronic glucose infusion yet gathered. We confirmed previous findings of transient insulin resistance, elevated *in vivo* β–cell function and an early wave of neogenesis. Our finding of a second wave of neogenesis provides further support for our previous

hypothesis of a late wave of acinar-to-islet transdifferentiation. Overall, the consistency between βIG model predictions and the prevailing literature suggest that this methodology may be a practical and reliable means of performing integrative and dynamic analysis of metabolic adaptation.

Acknowledgments

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Appendix

The equations for the βIG model (augmented to include exogenous glucose supply) are as follows.

$$dG/dt = R_0 - (E_0 + S_I I)G + G_{inf} \quad (1)$$

$$dI/dt = \beta\sigma G^2/(\alpha^2 + G^2) - kI \quad (2)$$

$$d\beta_n/dt = (\text{Replication} - \text{Death} + \text{Neogenesis})\beta_n \quad (3)$$

where G, I, β, β_n and t represent plasma glucose, insulin, β–cell mass, β–cell number and time respectively. R_0 and E_0 are hepatic glucose production at zero insulin and glucose effectiveness at zero insulin respectively. S_I is insulin sensitivity and G_{inf} is the glucose infusion rate. σ is the maximal rate at which a single β–cell can secrete insulin while α represent the sensitivity of insulin secretion to glucose (half max), and k is the insulin clearance constant. Full development of these equations can be found in Topp *et al.* [15].

Estimation of insulin sensitivity. Mean insulin sensitivity was calculated from equation 1 using mean glucose and insulin values for each group at each time point by assuming: 1) $dG/dt = 0$ at the time of the sample and 2) a constant, error free, value for R_0 and E_0.

The standard error of the estimate of insulin sensitivity in each group at each time point was calculated by the Taylor expansion method of equation 1 incorporating the standard errors of the glucose and insulin measurements.

$$S_I = (R_0 - EG + G_{inf})/(GI) \quad (4)$$

Estimation of β–cell function. β–cell function (σ) was calculated from equation 2 using mean glucose, insulin and β–cell mass values for each group at each time point assuming: 1) dI/dt = 0 at the time of the sample and 2) a constant, error free, value for α and k. Again the standard error of the estimate of β–cell function in each group at each time point was calculated by the Taylor expansion method of equation 2 incorporating the standard errors of the glucose, insulin and β–cell mass measurements.

$$\sigma = kI(\alpha^2 + G^2)/(\beta G^2) \quad (5)$$

Estimation of net-neogenesis. Since equation 3 contains an implicit assumption of constant size of individual β–cells, calculation of net-neogenesis required the incorporation of cell size to equation (3). Briefly, β–cell mass (β) is dependent on the number (β_n) and the size of pancreatic β–cells (β_s):

$$\beta = (\beta_s)(\beta_n). \quad (6)$$

The rate of change (d/dt) of β–cell mass can be expressed as a chain rule expansion of equation (6):

$$d\beta/dt = (d\beta_s/dt)(\beta_n) + (d\beta_n/dt)(\beta_s) \quad (7)$$

Dividing through by β and substituting in equation (3) for the rate of change of β–cell number we arrive at:

$$(d\beta/dt)/\beta = (d\beta_s/dt)/(\beta_s) + \text{Replication} - \text{Death} + \text{Neogenesis} \quad (8)$$

Since net-neogenesis (neogenesis – death) occurs on the same time scale as β–cell mass and β–cell size dynamics, one can not assume $d\beta/dt = 0$ or $d\beta_s/dt = 0$.

$$\text{Net-Neogenesis} = (d\beta/dt)/\beta - (d\beta_s/dt)/(\beta_s) - \text{Replication} \quad (9)$$

The dynamics of β, dβ/dt, β_s and $d\beta_s/dt$ were determined via simple curve fits (nonlinear least squares algorithm, MLAB, Civilized Software Inc) of β–cell mass and β–cell size

data. Since BrdU labels β–cells entering into S-phase during a 6 hour window, β–cell replication rates (% per day) were assumed to be 4 fold the percent of BrdU positive β–cells. Replication rate data from the saline infused group were fit with a simple linear equation; however, the dynamics of β–cell replication in the glucose infused animals was complex and not well fit by any simple polynomial or sigmoid equation. Thus replication rates from this group were determined by an optimal segments smoothing algorithm.

Normal parameter values. Normal, rat specific, parameter values used in this study are; $R_0 = 892.8$ mg dl^{-1} day^{-1} [43], $E = 1.44$ day^{-1}, [43], $\alpha = 20000$ mg^2 dl^{-2} [43], $k = 432$ day^{-1} [43]. G_{inf} was calculated to be 10435 mg dl^{-1} day^{-1} based on 50% dextrose infusion at 2ml/hr and an assumed 25% of mass glucose volume of distribution space.

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CHAPTER 3: DIABETES IN ZUCKER RATS

The Dynamics of Insulin Sensitivity, β-cell function, and β-Cell Mass During the Development of Diabetes in fa/fa Rats.

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Abstract

Both male Zucker Fatty (mZF) and low fat fed female Zucker Diabetic Fatty (fZDF-lf) rats are obese but remain normoglycemic. Male ZDF (mZDF) and high fat fed female ZDF rats (fZDF-hf) are also obese, but develop diabetes between 7 and 10 weeks of age. Although these models have been well studied, the mechanisms governing the adaptations to obesity in the normoglycemic animals, and the failure of adaptation in the animals that develop diabetes, remain unclear. Here we use quantitative morphometry and our recently develop βIG model to elucidate the dynamics of insulin sensitivity (S_I), β-cell secretory capacity (β_{sc}) and β-cell mass (β_m) in these four animal models. Both groups that remained normoglycemic with increasing obesity (mZF, fZDF-lf) exhibited increased β_m and constant β_{sc} in response to a falling S_I. In rats that developed hyperglycemia (mZDF, fZDF-hf), there was a greater reduction in S_I and slower expansion of β_m, with constant β_{sc}. β_{sc} decreased after glucose levels rose above 20 mmol. Taken together these data suggest that excessive insulin resistance and insufficient β-cell mass adaptation play a primary role in the pathogenesis of diabetes while insulin secretory defects occur secondary to hyperglycemia in these animals.

Introduction

Zucker Fatty (ZF) rats possess a leptin receptor defect that results in obesity and insulin resistance [1]. However, these animals remain normoglycemic via compensatory hyperinsulinemia. Successive inbreeding of the most glucose intolerant ZF rats led to the development of Zucker Diabetic Fatty (ZDF) rats [1]. Male ZDF rats spontaneously develop overt hyperglycemia between 6 and 12 weeks of age while female ZDF rats only become hyperglycemic when placed on a high fat diet [1, 2]. Although commonly studied there is much uncertainty regarding the dominant mechanisms governing the normal and pathological adaptations to obesity in this model.

There is little doubt that β-cell mass dynamics contribute to the development of compensatory hyperinsulinemia in ZF rats. Several studies have shown β-cell mass to be elevated in these animals relative to lean controls [3, 4]. Further, β-cell mass has been shown to expand dramatically during the development of obesity [3]. The contributions of β-cell function (defined as secretory capacity per unit β-cell mass) to the development of compensatory hyperinsulinemia are less clear. Some studies have found β-cell function to be either left shifted or elevated in islets from ZF rats [5, 6, 7]. However, recent perfused pancreas studies have shown β-cell function to be similar in ZF and lean Zucker rats [3, 4]. Studies of β-cell function in other animal models has also produced equivocal results. Chronic glucose infusion and partial pancreatectomy studies have clearly shown β-cell function to be plastic and responsive to acute changes [8, 9]. However, other studies have shown chronic hypersecretion to be unsustainable (β-cell exhaustion) [10]. Overall, the relative contributions of β-cell mass and function to the hyperinsulinemia of obesity remain unresolved.

In the pre-diabetic state, there is little to separate the male ZDF from the ZF rat. Both are obese and insulin resistant. They display similar β-cell function, mass, and replication rates [3]. However, by 12 weeks of age, male ZDF rats display reduced levels of both β-cell mass and β-cell function [3]. The known effects of hyperglycemia and dyslipidemia on

muscle glucose uptake would suggest that adult male ZDF rats are more insulin resistant than male ZF rats [11, 12]. However, a direct comparison of insulin sensitivity levels between these animals has yet to be reported. It has been widely suggested that the transition from normoglycemia and pre-diabetes to overt hyperglycemia in male ZDF rats occurs via lipotoxicity [13]. Pre-diabetic ZDF rats have been shown to display high levels of plasma FFA and high susceptibility to β-cell lipotoxicity [13]. Together these studies suggest that abnormal lipid buffering causes exacerbation of insulin resistance, decreased insulin secretion and increased β-cell apoptosis. However, the longitudinal data required to support this evidence has yet to be reported.

Relatively little is know about female ZDF rats. On a low fat diet they have been shown to display lower plasma FFA levels than male ZDF rats, suggesting improved lipid partitioning and reduced susceptibility to lipotoxicity [13]. Recently, the high fat fed female ZDF rat has been proposed as an animal model of diet induced type 2 diabetes [2]. The mechanisms responsible for the initiation and exacerbation of hyperglycemia in these animals remain largely unexplored.

Much of the controversy surrounding the relative contributions of insulin resistance, β-cell function and β-cell mass to the pathogenesis of diabetes in these animals is due to a lack of integrative longitudinal data. Traditional methodologies for estimating insulin sensitivity and pancreatic insulin secretory capacity (the hyperinsulinemic clamp and the perfused pancreas) are expensive, time consuming, require special experimental equipment, and would necessitate a different cohort of animals for each metabolic index and point in time. While mathematical models such as the Minimal and HOMA models have vastly improved the practicality of measuring insulin sensitivity and pancreatic insulin secretory capacity in humans, they have not been adapted to the most commonly used laboratory animals (rats and mice) [14, 15]. Recently we developed a mathematical model of coupled β-cell mass, insulin and glucose (βIG) dynamics [16]. Similar to the HOMA method this model was designed to estimate insulin sensitivity and β-cell function from fasting glucose and insulin data. In addition, this model is capable of

estimating net neogenesis (neogenesis – death) from estimates of β-cell mass and β-cell replication rates. Using human parameter values, βIG model estimates of insulin sensitivity and β-cell function showed strong correlation to the HOMA and Minimal models in a large human data set spanning the range of type 2 diabetes [17]. Adjusting to rodent specific parameter values, βIG model estimates of insulin sensitivity, β-cell function, and net neogenesis showed strong agreement with previously published *in vivo* measures of these indices during chronic glucose infusion in Sprague-Dawley rats [8].

Here we use histological methods and the βIG model to estimate the full dynamics of insulin sensitivity, β-cell function, β-cell mass, β-cell replication rates and net neogenesis in two animal models of obesity (male ZF and female ZDF rat) and two animal models of type 2 diabetes (male ZDF and high fat fed female ZDF).

Methods

Animals. Thirty-two male ZF, 32 male ZDF, and 54 female ZDF rats were obtained from Genetic Models incorporated (Indianapolis, IN). Animals were housed individually with free access to food and water. Male rats were fed Purina 5008 (16.5% fat by calorie) while female rats were randomly assigned either low fat (Purina 5001, 12% fat by calorie) or high fat (GMI 13004, 47.9% fat by calorie) chow. Weekly blood samples (~0.1 ml) were taken from the saphenous vein. Eight animals per group were sacrificed bi-weekly. Six hours prior to sacrifice, rats were given an i.p. injection of 5-bromo-2'deoxyuridine (100 mg/kg BrdU, Sigma-Aldrich, Oakville, ON, Canada). Glucose, insulin, β-cell mass and BrdU data from the male ZDF rats were previously reported [18]. All procedures were performed in accordance with the standards set forth by the Canadian Council on Animal Care and were approved by the Animal Care Committee at Simon Fraser University.

ASSAYS. Plasma samples were centrifuged and stored at -20 °C until assayed for glucose (Glucose Trinder, Sigma Diagnostics, St. Louis, MO.) and insulin (rat insulin ELISA, Crystal Chem Inc., Downers Grove, IL.).

Pancreatectomy. Pancreatectomies were performed as previously described (14). Tissue samples were cut in two then placed in a fixative (mixture of 75 ml water, 25 ml formaldehyde (37%), and 5 ml glacial acetic acid) for 48 hours at room temperature. They were washed three times in phosphate buffered saline (PBS) (pH 7.4), then left in PBS at 4^0C overnight. Samples were washed three times in 70% alcohol, placed in cassettes and embedded in paraffin. Five serial sections (4 μm) from each block (2 sets per animal) were cut using an Olympus microtome (CUT 4060; Carsen Group, Markham, ON, Canada) and were mounted on poly-L-lysine coated slides.

Staining. Two serial sections from each block were stained and analyzed. The first slide was stained with anti-insulin antibody while the second was double-stained with anti-insulin and anti-BrdU antibodies. All sections were dewaxed in xylene, dehydrated in petroleum ether, and incubated in 0.3% hydrogen peroxide in methanol for 30 min. Sections were then washed in PBS and incubated in 10% lamb serum in PBS for an additional 30 min. Sections were serially incubated with guinea pig anti-insulin antibody (1:1000) (Dako Diagnostics, Mississauga, ON, Canada) at 37^0 C for 30 min, biotinylated anti-guinea pig antibody (1:500) (Vector Laboratories, Burlington, ON, Canada) for 1 hr, and avidin/biotin horseradish peroxidase complex (ABC-HP) (1:1000) (Vectastain Elite ABC Kit; Vector Laboratories) for 1 hr at room temperature. Samples were then developed in 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (Sigma-Aldrich, Oakville, ON, Canada) for 10 min. Sections were washed several times with PBS between incubations. After DAB development, sections were washed in running tap water, counterstained with hematoxylin (Harris, Sigma-Aldrich) and coverslipped with Permount mounting media (Fisher Scientific , Nepean, ON, Canada).

The sections double stained for bromodeoxyuridine (BrdU) and insulin were dewaxed and hydrated as described above and then incubated with anti-bromodeoxyuridine

monoclonal antibody with nuclease (Amersham Pharmcia Biotech, Baie d'Urfé, Quebec, Canada) for 30 min at 37^{0}C. Sections were washed with PBS and incubated serially with biotinylated goat anti-mouse antibody (Vector) for 1 hr and ABC-HP (1:1000) (Vector) for 1 hr at room temperature. Slides were washed in PBS between incubations. Sections were developed in DAB solution for 2-3 min. Slides were washed with PBS and incubated with guinea pig anti-insulin antibody (DAKO Diagnostics) for 30 min at 37^{0}C. Next they were serially incubated with biotinylated anti-guinea pig antibody (1:500) (Vector) for 1hr and avidin/biotin alkaline phosphatase complex (Alkaline Phosphatase Standard Vectastain ABC Kit, Vector Laboratories) for 1 hr at room temperature. PBS washes were carried out between incubations. Sections were developed in Dako Fuchsin Substrate-Chromogen System for 10 min (Dako Diagnostics). Slides were then washed in water, counterstained with hematoxylin and coverslipped with Permount.

Quantitative morphometry. β-cell mass was determined via quantitative analysis of the insulin antibody stained slides. An image analysis system consisting of an Olympus light microscope (Model BX40; Carsen Group) attached to a Sony color video camera (Model DXC-950; Sony, Japan) and MetaVue image analysis software (Universal Imaging Corporation, Downingtown, PA) was utilized to estimate the β-cell and non-β-cell area on each slide. A point counting system was used to estimate the ratio of adipose to non-adipose tissue on each slide. The ratio of β-cell to non-β-cell tissue was determined for each animal then multiplied by the pancreatic mass to generate an estimate of total β-cell mass. Ten slides from each group were used to determine the relationship between β-cell number and β-cell area. These correlations were then used to estimate the total number of pancreatic β-cells on each slide. Double-stained slides were then manually analyzed to determinate the total number of BrdU positive β-cells, and combined with the β-cell number data to determine the percentage of replicating β-cells.

βIG model analysis. The equations for the βIG model (13) are as follows.

$$dG/dt = R_0 - (E_0 + S_I I)G \quad (1)$$

$$dI/dt = \beta_m \beta_{sc} G^2/(\alpha^2 + G^2) - kI \quad (2)$$

$$d\beta_m/dt = (\text{Replication} - \text{Death} + \text{Neogenesis})\beta_m \quad (3)$$

where G, I, β_m and t represent plasma glucose, insulin, β-cell mass, and time respectively. R_0, E_0, and S_I represent the maximal rate of hepatic glucose output, glucose effectiveness at zero insulin, and insulin sensitivity respectively. β_{sc}, α and k represents the β-cell secretory capacity (maximal rate of secretion per unit β-cell mass), the glucose level that induces half maximal insulin secretion rates, and the insulin clearance rate.

Equation 1 was utilized to estimate insulin sensitivity for each animal at each point in time. Briefly, dG/dt was assumed to be zero (i.e. the minute-to-minute changes were negligible relative to the 6 week time frame of the study), and constant values were assigned to R_0 and E_0 based on the literature. The measured glucose and insulin levels could then be placed into equation 1 and used to estimate S_I.

$$S_I = (R_0 - EG)/(GI) \quad (4)$$

Equation 2 was utilized to estimate β-cell secretory capacity (β_{sc}) from group mean G, I and β_m data. As for glucose, dI/dt was assumed to equal zero and constant "normal" values were assigned to α and k. The group mean levels of G, I and β_m were then placed into equation 2 to estimate β-cell secretory capacity (β_{sc}) at each time point. β-cell mass levels for 7, 9, and 11 weeks of age were determined by averaging the levels measured at the preceding and following time points.

$$\beta_{sc} = kI(\alpha^2 + G^2)/(\beta_m G^2) \quad (5)$$

The standard error of β_{sc} was estimated via the Taylor expansion method for propagating the standard errors from measured G, I and β_m data at each point in time.

Equation 3 was utilized to estimate net-neogenesis (neogenesis – death). Since β-cell mass dynamics occur on a much slower time scale (days to weeks) relative to glucose and insulin dynamics (minutes) $d\beta_m/dt$ could not be assumed to be zero. Thus $d\beta_m/dt$ was

approximated from the β-cell mass data ($\Delta\beta_m/\Delta t$) and assigned to the time point in-between the measured points (i.e. at 7, 9 and 11 weeks of age). Propagation of error was used to estimate the uncertainty surrounding these calculated values. The percentage of β-cells replicating per day were assumed to be 4 times the percent of BrdU positive cells (as BrdU is injected 6 hour prior to termination). β_m and replication rates at 7, 9, and 11 weeks of age were estimated by averaging the data measured at 6, 8, 10, and 12 weeks of age and propagation of error was used to estimate the standard errors on these calculated values. Net-neogenesis was then calculated with the following formula:

$$(\Delta\beta_m/\Delta t)/\beta_m - \text{Replication} = \text{Neogenesis} - \text{Death} = \text{Net Neogenesis.} \quad (6)$$

The standard error about net neogenesis was estimated via propagating the standard errors of ($\Delta\beta_m/\Delta t$), β_m, and replication rate data.

Statistics. Data are presented as mean ± standard error unless otherwise noted. Comparisons between points in time and treatment groups were performed via a repeated measured two-way ANOVA with post hoc Tukey analysis. All statistical calculations were performed using SAS Version 7.0 (The SAS Institute, Cary, NC).

Results

High fat fed female ZDF rats (HF-fZDF) consumed fewer calories than low fat fed female ZDF (LF-fZDF) rats (93.4 ± 1.1 vs. 97.4 ± 0.6 cal/day, $p < 0.05$) despite being fed a high calorie chow diet (4.9 vs. 3.9 cal/g) *ad libitum* (Figure 3-1). Body weight differed only slightly between groups of female rats. At the beginning of the study, male ZDF (mZDF) rats consumed fewer calories, than male ZF (mZF) rats, while displaying similar water intake and body weight. The development of diabetes in mZDF rats was associated with increased food and water intake as well as reduced weight gain. Male ZDF rats ingested more calories and put on more body weight than female ZDF rats. In addition, despite similar levels of hyperglycemia at 12 weeks of age (Figure 3-2), HF-fZDF rats had less severe hyperphagia and glucosuria as compared to mZDF rats.

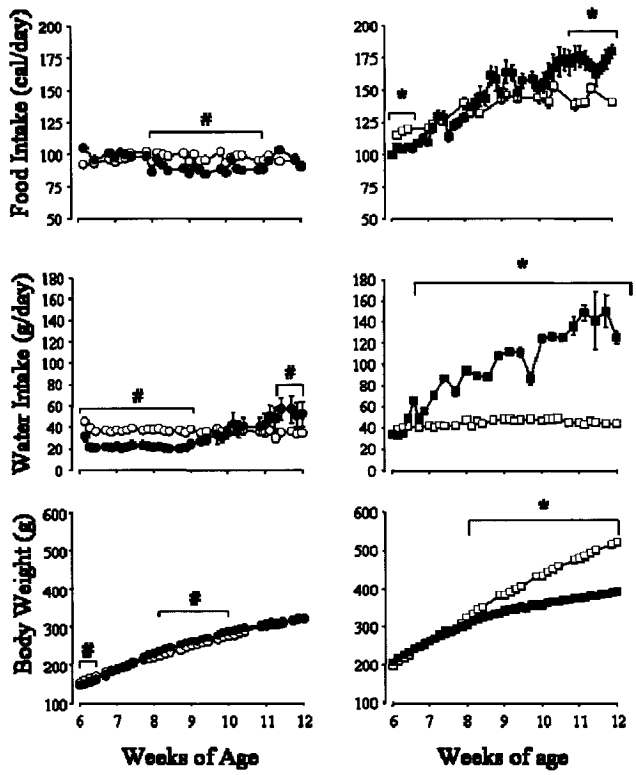

Figure 3- 1 Food intake, water intake, and body weight in LF-fZDF (white circles) and HF-fZDF (black circles) rats, as well as mZF (white squares) and mZDF (black squares) rats. # $p < 0.05$ HF-fZDF vs LF-fZDF, * $p < 0.05$ mZDF vs mZF.

Glucose and insulin dynamics for all four groups of animals are displayed in Figure 3-2. LF-fZDF rats developed moderate hyperglycemia and hyperinsulinemia over the course of this study. HF-fZDF rats developed overt hyperglycemia between 8 and 11 weeks of age while insulin levels display a biphasic pattern; increasing for the first three weeks then decreasing thereafter. Overt hyperglycemia (>20 mM) preceded the decrease in insulin levels. Male ZF rats remained normoglycemic throughout while displaying a 3-4 fold increase in plasma insulin levels. Male ZDF rats displayed 3 weeks of rising glucose levels accompanied by biphasic insulin dynamics. Similar to HF-fZDF rats, overt hyperglycemia preceded the decrease in insulin levels in mZDF rats. Male rats had a higher insulin level relative to female rats at the beginning of the study. Also, the decrease in insulin levels was less pronounced and was initiated at a higher glucose level in HF-fZDF rats relative to mZDF rats.

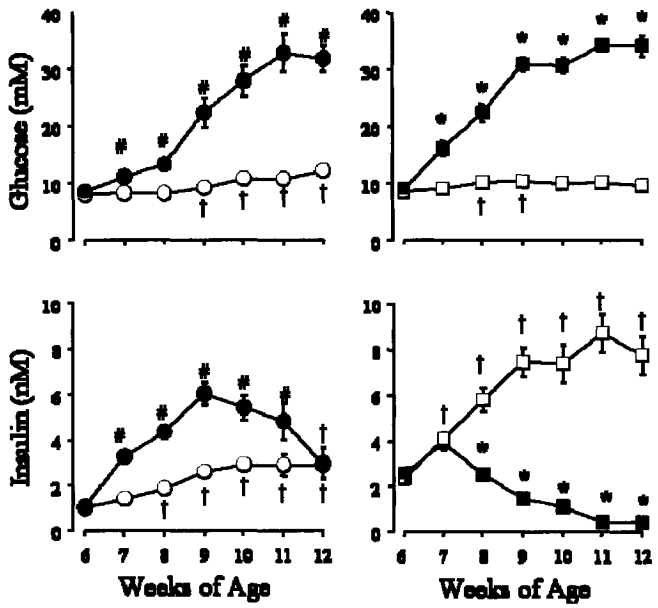

Figure 3- 2 Plasma glucose and insulin dynamics in LF-fZDF (white circles) and HF-fZDF (black circles) rats, as well as mZF (white squares) and mZDF (black squares) rats. ‡ $p < 0.05$ t vs t = 0, # $p < 0.05$ HF-fZDF vs LF-fZDF, * $p < 0.05$ mZDF vs mZF.

Figure 3-3 shows the β-cell mass and replication rate data for all 4 groups of Zucker rats. Both groups of female ZDF rats displayed a two- to three-fold increase in β-cell mass. This early rapid expansion was supported by high rates of β-cell replication (~8% per day) that fell to a moderately elevated level of ~4% per day by the end of the study. Male ZF rats displayed a 3 to 4-fold increase in pancreatic β-cell mass between 6 and 10 weeks of age which was due, at least in part, to elevated rates of β-cell replication (~8% per day) that subsided to moderately elevated levels (~4% per day) by the end of the study. During the first two weeks of study, mZDF rats demonstrated a slow rate of β-cell mass expansion relative mZF rats (6.9 ± 0.5 vs. 2.0 ± 1.4 % per day, $p < 0.05$). This was followed by a ~50% reduction in β-cell mass between 8 and 12 weeks of age ($p < 0.05$). These β-cell mass dynamics were associated with reduced rates of β-cell replication throughout the study. Male ZDF rats displayed a higher β-cell mass at the beginning of the study as compared to female ZDF rats, but this difference disappeared by 8 weeks of age and was reversed by 12 weeks of age. Replication rates were lower in mZDF rats throughout. Also, it should be noted that, despite similar end study glucose levels, HF-fZDF rats did not display any significant reduction in β-cell mass. This discrepancy may

be due to the fact that mZDF rats develop overt hyperglycemia two weeks earlier than HF-fZDF rats.

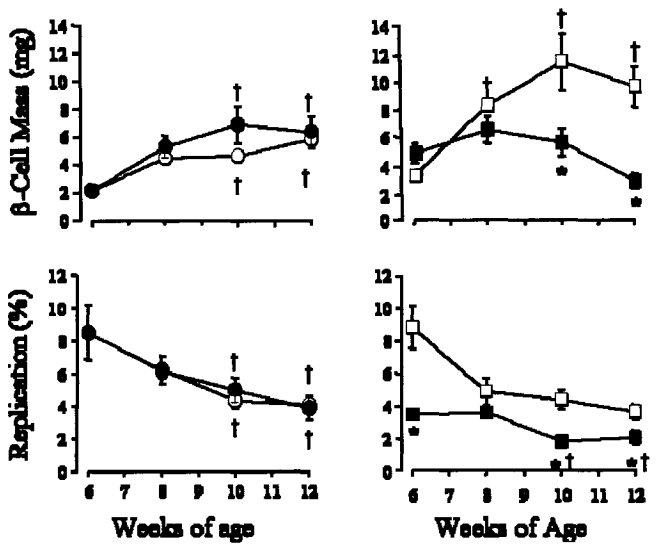

Figure 3- 3 β-cell mass and β-cell replication rate dynamics in LF-fZDF (white circles) and HF-fZDF (black circles) rats, as well as mZF (white squares) and mZDF (black squares) rats. ‡ $p < 0.05$ t vs t = 0, # $p < 0.05$ HF-fZDF vs LF-fZDF, * $p < 0.05$ mZDF vs mZF.

βIG model estimates of insulin sensitivity, β-cell secretory capacity and net-neogenesis are presented in Figure 3-4. LF-fZDF rats displayed a ~75% reduction in insulin sensitivity (S_I) while β-cell secretory capacity (β_{sc}, an index of the maximal rate of glucose induced insulin secretion per unit β-cell mass) remained constant. There were no significant changes in net neogenesis in LF-fZDF rats. HF-fZDF rats quickly became more insulin resistant than LF-fZDF rats. β-cell secretory capacity was constant in HF-fZDF rats prior to the development of hyperglycemia, but decreased thereafter. Net-neogenesis was similar in both groups of female rats. Male ZF rats displayed a ~70% decrease in S_I between 6 and 8 weeks of age while β_{sc} remained constant and net neogenesis decreased. Male ZDF rats were more insulin resistant than mZF rats during the initiation (week 7) and exacerbation (weeks 9-11) of hyperglycemia. β-cell secretory capacity remained constant during the initiation of hyperglycemia, but decreased thereafter. Net-neogenesis did not differ between mZDF and mZF. Male rats were more insulin resistant than female rats. Prior to the development of hyperglycemia β_{sc} did not

differ between mZDF and fZDF rats, however, male rats displayed more marked reductions in β_{sc} following the development of overt hyperglycemia.

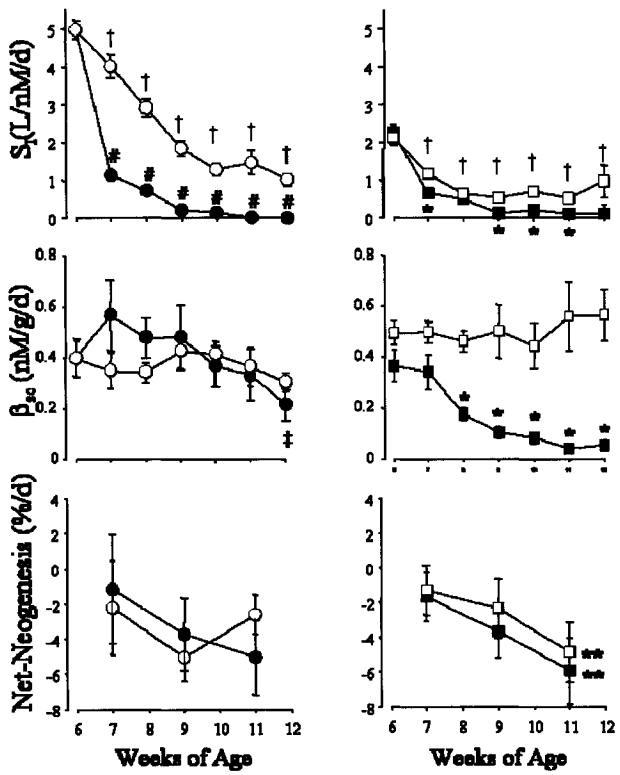

Figure 3- 4 βIG model estimates of the dynamics of insulin sensitivity, β-cell secretory capacity, and net-neogenesis in LF-fZDF (white circles) and HF-fZDF (black circles) rats, as well as mZF (white squares) and mZDF (black squares) rats. ‡ $p < 0.05$ t vs t = 0, # $p < 0.05$ HF-fZDF vs LF-fZDF, * $p < 0.05$ mZDF vs mZF.

Overall, these data suggest that in obese control animals, β-cell mass adaptation offset decreasing insulin resistance such that their disposition index (Figure 3-5) remained relatively constant. However, excessive insulin resistance and insufficient β-cell mass adaptation led to an initial decrease in the disposition index and the initiation of hyperglycemia in both obese diabetic animals. This hyperglycemia was followed by progressive insulin secretory defects that further reduced the disposition index and exacerbated hyperglycemia (Figure 3-5).

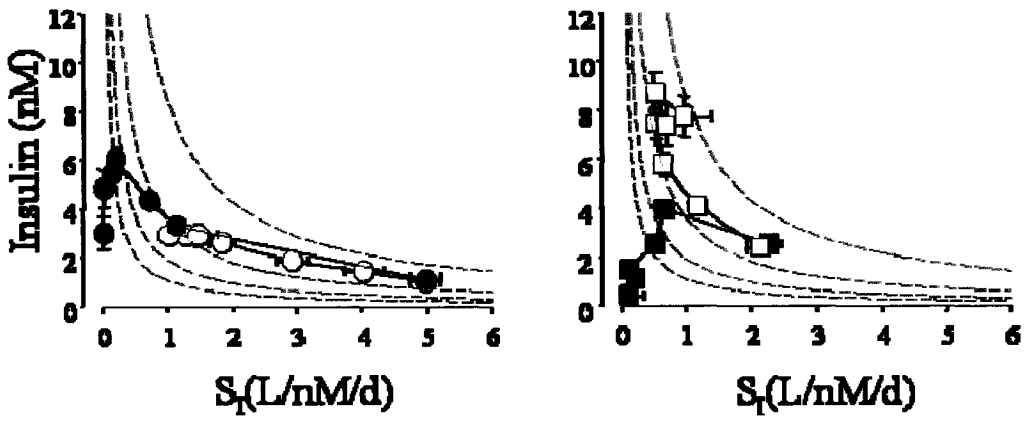

Figure 3- 5 (A) Dynamics of the disposition index for during the course of study in LF-fZDF (white circles) and HF-fZDF (black circles) rats, as well as (B) mZF (white squares) and mZDF (black squares).

Discussion

Adaptation to obesity and the pathogenesis of type 2 diabetes are complex processes characterized by time dependent changes in several key metabolic pathways. Here we utilized the tools of quantitative morphometry and mathematical modeling to estimate the dynamics of insulin sensitivity, β-cell function and β-cell mass during normal and pathological adaptation to obesity. In both obese control groups we found adaptation to insulin resistance occurs via increased β-cell mass while β-cell function remained constant. During the initiation of hyperglycemia, mZDF and HF-fZDF rats displayed excessive insulin resistance and insufficient β-cell mass adaptation. Progressive reductions in β-cell function occurred after overt hyperglycemia (20 mmol) was established. Together this suggests that excessive insulin resistance and abnormal β-cell mass dynamics play primary roles in the pathogenesis of diabetes while insulin secretory defects occur secondary to glucotoxicity.

Sufficient adaptation to obesity. Male ZF rats were more insulin resistant than female ZDF rats throughout the study. Between 6 and 12 weeks of age, insulin sensitivity dropped significantly in both obese control animals. This was accompanied by increased β-cell mass and constant β-cell function. While this suggests that β-cell function does

not respond to the development of insulin resistance, it should be noted that 1) these animals were moderately obese and insulin resistant at the beginning of the study and 2) estimates of β-cell function reported here are ~20 fold higher than βIG model estimates of β-cell function previously reported in adult Sprague-Dawley rats [8]. This suggests that β-cell function may have adapted maximally prior to 6 weeks of age.

Our finding of increased β-cell mass in obese control animals is consistent with other animal and human studies including other studies in male ZF rats [3, 4, 19, 20]. However, the literature on β-cell function in obesity is less clear. Glucose infusion and partial pancreatectomy studies have clearly demonstrated acute adaptation of β-cell function in response to increased insulin demand [8, 9]. However, the concept of β-cell exhaustion would suggest that β-cell hypersecretion may not be sustainable in the long run [10, 21]. Studies of function in obese Zucker rats have also generated mixed results. Some studies have reported a "left shift" in glucose induced insulin secretion in islets from humans and Zucker rats [5, 22]. Other studies have found β-cell function not to differ between obese and lean Zucker rats [3, 4]. Finally, it should be noted that, due to the *in vivo* nature of our index of β-cell function (β_{sc}), differences between Sprague-Dawley and Zucker rat values for β_{sc} may reflect differences in insulin clearance and/or non-glucose secretagogues rather than adaptation of glucose induced insulin secretion *per se*. However, it is difficult to imagine that the 20 fold difference in β_{sc} for Sprague-Dawley and obese Zucker rats can be fully accounted for by differences in insulin clearance rates. Overall, these data support the concept of a feedback loop between β-cell mass and insulin sensitivity and suggest that β-cell function adapted maximally prior to 6 weeks of age.

Insulin resistance during the development of hyperglycemia. At the beginning of the study, insulin sensitivity did not differ between diabetes prone and sex matched obese controls. During the initiation of hyperglycemia, both groups of obese diabetic animals developed greater insulin resistance than their respective obese controls. Several hyperinsulinemic clamp studies have shown both the male ZDF and male ZF to be insulin

resistant, however, this is the first study to compare the dynamics of insulin sensitivity in these animals. These observations suggest that additional insulin resistance contributes to the development of hyperglycemia rather than only being a result of it. Although we did not report plasma lipid dynamics here, Lee *et al.* found FFA levels to increase in male vs. female ZDF rats two weeks prior to the development of hyperglycemia [13]. This suggests that the excessive insulin resistance observed during the initiation of hyperglycemia may be due to lipotoxicity.

β-cell function during the development of hyperglycemia. β-cell function did not differ between groups until glucose levels rose above 20 mmol. This suggests that the progressive insulin secretory defects observed in these animals occurred secondary to glucotoxicity and thus do not play a primary role in the pathogenesis of diabetes in these animals. Other studies have shown β-cell function to be normal or elevated in pre-diabetic ZDF rats [3, 6, 23], however, this is the first study to show β-cell function to remain constant during the initiation of hyperglycemia. It should be noted that while several studies have suggested that ZDF rats display a primary defect in β-cell function, most of these studies have used lean islets as a control [24, 25, 26]. Thus it is unclear if these previously reported defects are common to the whole Zucker family, or if they occur only in ZDF rats. It is interesting to note that, despite previous reports of increased FFA in pre-diabetic ZDF rats and increased susceptibility of ZDF islets to lipotoxicity, β-cell function did not decrease in either diabetes prone strains until glucose levels reached ~20 mmol [13, 27]. These findings are consistent with the argument that hyperglycemia is required element of lipotoxicity [28].

β-cell mass dynamics during the development of hyperglycemia. Despite the added insulin resistance incurred by these animals, β-cell mass adaptation remained similar to, or slower than, rates observed in obese non-diabetic animals. Also, β-cell mass decreased in the male but not high fat fed female ZDF rat and did so at a much higher glucose level than was associated with the initiation of insulin secretory defects. Our observation of reduced β-cell mass in overtly diabetic ZDF rats is consistent with other

studies [3], however, this is the first study to show that abnormal mass dynamics contribute to the initiation of hyperglycemia in these animals. Interestingly, Pick *et al.* found β-cell replication rates to be similar in ZDF and ZF rats [3]; suggesting that male ZDF rats display elevated levels of β-cell apoptosis. Also, previous findings of increased FFA in pre-diabetic male ZDF rats and increased susceptibility of ZDF islets to lipoapoptosis [27] would suggest that high rates of β-cell death would play a primary role in the development of hyperglycemia. Here we found β-cell apoptosis to be similar in all four animal models, while β-cell replication rates were reduced in male ZDF rats. It should be noted that we calculated net β-cell death (neogenesis – death) leaving open the possibility that increased rates of β-cell death in male ZDF rats are "hidden" by increased rates of neogenesis.

Lipid partitioning during the development of hyperglycemia. It is interesting to note that food intake and body weight were similar or reduced in diabetic animals during the initiation of hyperglycemia. This suggests that the additional insulin resistance observed in these animals was not likely a result of additional adiposity. One possible explanation for this is a defect in lipid partitioning that results in increased triglyceride accumulation in non-adipose tissue [29].

Summary. This study has used a combined mathematical and histological approach to generate a complete analysis of the normal and pathological adaptations to obesity in Zucker rats. As a result we were able to show that both excessive insulin resistance and insufficient β-cell mass adaptation contribute to the initiation of hyperglycemia while insulin secretory defects appear to be a secondary consequence of glucotoxicity.

Acknowledgments

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CHAPTER 4: REGULATION OF β-CELL MASS *IN VIVO.*

Effects of Acute Hyperglycemia on β-Cell Mass Dynamics in Vivo .

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Abstract

Aims/hypothesis. While in vitro studies have shown clear relationships between glucose levels and β-cell replication and death rates, the role of blood glucose levels in the regulation of b-cell mass dynamics *in vivo* remains much debated. Here we quantify rates of β-cell replication, death, and neogenesis following 24 hours at various levels of hyperglycemia.

Methods: A modified hyperglycemic clamp protocol is used to clamp glucose for 24 hours at basal, 15, 20, 25 or 35 mmol in cohorts of young Sprague-Dawley rats (n = 40). Pancreata were removed for determination of β-cell mass (computerized morphometry), replication (via BrdU), death (via TUNNEL), and neogenesis rates (quantification of "neogenic morphologies").

Results. Relative to un-infused control animals, replication rates were reduced in the 15 and 20 mmol groups but unchanged in the 25 and 35 mmol groups. However, if one excludes the non-infused control cohort, mean β-cell replication rates displayed a clear linear relationship to mean blood glucose ($r^2 = 0.98$, $p < 0.05$). The percentage of TUNNEL positive β-cell was elevated in the 35 mmol cohort of animal, but did not differ from un-infused basal glucose animal in any other group. Despite the observation of reduced replication or increased death rates in all hyperglycemic cohorts, β-cell mass did not differ between groups. This, coupled to the observation of "focal" areas implies hyperglycemia induced β-cell neogenesis and/or transdifferentiation.

Conclusions/interpretation. Our observation of a strong linear relationship between glycemia and β-cell replication rates in all hyperglycemic animals suggests that glucose does regulate β-cell replication rates *in vivo*. However, the fact β-cell replication rates in the non-infused animals did not fit on this line may imply the existence of a second regulatory signal/process that is active in glucose infused, but not control animals. Also, our findings are consistent with the concepts of gluco-apoptosis and glucose induced neogenesis/transidifferentiation.

Introduction

Although still somewhat controversial [1], it is becoming increasingly clear that β-cell mass dynamics contribute significantly to both the normal adaptation to obesity as well as the pathogenesis of type 2 diabetes [2, 3, 4]. Several human and animal studies have shown β-cell mass to be elevated in obesity and responsive to changes in insulin sensitivity [2, 5, 6, 7]. Similarly, several human and animal studies have shown β-cell mass to be reduced, relative to weight matched controls, in type 2 diabetes [3, 5, 8]. While it is not yet possible to measure β-cell mass longitudinally, cross-sectional animal data suggests that β-cell mass dynamics display an inverted U, or "Starling Curve" similar to that displayed by blood insulin levels during the pathogenesis of type 2 diabetes [9, 10, 11]. It has been widely suggested that the progressive reductions in β-cell mass occur as a secondary consequence of the hyperglycemia, dyslipidemia, and amyloid plaque formation that characterize the late stages of type 2 diabetes [12, 13, 14]. However, there is some evidence to suggest that slow expansion of the β-cell mass during the development of hyperglycemia can contribute significantly to the initiation of hyperglycemia/dyslipidemia and thus the pathogenesis of type 2 diabetes [8, 15]. Together these and other findings have made the β-cell mass an attractive target for the prevention and treatment of type 2 diabetes [16, 17]. However, the signals and mechanisms which regulate β-cell mass dynamics remain unclear and much debated.

Of the proposed signals, glucose has received the most attention. Blood glucose levels represent a signal that is both directly affected by the development of insulin resistance and is easily detected by pancreatic β-cells. *In vitro* studies have shown moderate hyperglycemia to stimulate β-cell mass expansion (via increased replication and decreased death) while extreme hyperglycemia caused β-cell mass to decrease (via inhibition of replication and stimulation of apoptosis) [18, 19, 20, 21, 22]. Incorporating this *in vitro* data into a mathematical model of β-cell mass, insulin and glucose dynamics, we were able to reproduce the "Starling curves" (insulin and β-cell mass) previously reported in the experimental literature [23]. While this data is consistent with the hypothesis that

glucose is a primary regulator of β-cell mass dynamics, the relationship between glycemia and β-cell mass dynamics, *in vivo*, has yet to be fully determined.

Several researchers have utilized the chronic glucose infusion protocol to quantify the effects of hyperglycemia on β-cell mass dynamics *in vivo* [24, 25, 26, 27, 28, 29]. While the vast majority of these studies found hyperglycemia to stimulate β-cell mass adaptation, these studies have reported too few data points (usually basal and extreme hyperglycemia) to fully quantify the relationship between glycemia and β-cell mass dynamics *in vivo*. Interpretation of this data is further clouded by the fact that constant glucose infusion induces transient hyperglycemia (~2 days) with lasting effects on β-cell mass adaptation [28, 27]. Cross-sectional analysis of the pathogenesis of type 2 diabetes has shown β-cell expansion to correspond to moderate hyperglycemia and β-cell contraction to occur during periods of overt hyperglycemia [11]. However, other studies have reported β-cell mass expansion in the absence of hyperglycemia [30, 31]. Also, several other metabolites and hormones (including insulin, lipids, insulin like growth hormone, incretin hormones and leptin) have been shown to stimulate β-cell adaptation [32, 33, 34, 27, 35]. Overall, the relationship between blood glucose levels and β-cell mass dynamics *in vivo* remains unclear and much debated.

In this study we develop a 24 hour hyperglycemic clamp protocol to quantify the effects of five glucose levels (basal, 15, 20, 25, and 35 mmol) on β-cell mass as well as the rates of β-cell replication, death, and neogenesis *in vivo*.

Methods

Animals. Six week old Sprague-Dawley rats (n = 40) were ordered from Charles River Inc. (Wilmington, MA) and were housed individually with free access to food and water, during a one week acclimation period. At 7 weeks of age, indwelling catheters were imbedded into the right carotid artery and left jugular vein. During 4 days of recovery, catheter patency was maintained with heparinized saline lock (100 IU Heparin/ml saline)

and daily flushing with 0.9% saline. Food was removed 12 hours prior to initiation of the experiment. A jacket and tether system was used to allow the rats free range about their cage while preventing chewing of the lines. All procedures were performed in accordance with the standards set forth by the Canadian Council on Animal Care and were approved by the Animal Care Committee at Simon Fraser University.

Surgery. Animals were anaesthetized with Ketamine (100mg/ml) and Rompun (20 mg/ml) mixed at a 2:1 ratio administered at a dose of 0.1 ml/100g im. Atropine (0.04 mg/kg sc) and Butorphanol (1.0 mg/kg) were also given prior to the implantation of jugular vein and carotid artery catheters (Intramedic PE50 with silastic tips attached on the intravenous side) as described previously (Bonner-Weir *et al.* 1989). The catheters were channelled subcutaneously and exteriorized at the nape of the neck. Following surgery, each rat received a single dose of antibiotic (Baytril, 5 mg/kg s.c.) and analgesic (Torbugesic, 1 mg/kg i.m.).

Clamp protocol. Each animal was randomly assigned to a clamp group of basal, 15, 20, 25, or 35 mmol glucose. At t = -60 and -30 min, blood samples were taken (0.05 ml) from the carotid artery for determination of basal glucose and insulin levels as well as percent cellular volume (PCV). At t = 0, a glucose bolus (0-1 ml of 50% dextrose) was administered into the jugular vein and followed by a continuous infusion of 50% dextrose (0-3 ml/hr). Arterial blood samples (0.05 mL) were taken every 30 min, spun down, and immediately assayed for plasma glucose concentration (Beckman Glucose Analyzer Type 2). Fifteen minutes after each blood sample, the glucose infusion rate was adjusted in an attempt to maintain the desired glucose level. The remaining plasma was frozen and assayed for insulin concentrations (Rat insulin ELISA kit, Crystal Chem Inc., Cowners Grove, Chicago IL) at a later date. Red cells were collected from each sample, suspended in 2-3 drops of Heparinized saline (100 IU/ml), kept on ice, and re-infused to the animals every 2 hours. In total 50 blood samples (2.5 ml) were taken during the 24 hours of infusion. PCV was calculated from the final blood sample to ensure that the animals had not become anaemic. Six hours prior to termination an ip injection of 5-bromo-

2'deoxyuridine (100 mg/kg BrdU, Sigma-Aldrich, Oakville, ON, Canada) was administered.

Pancreatectomy. Pancreatectomies were performed as described previously (Finegood *et al.* 2001). Briefly, the spleen, pancreas, and duodenum were isolated and removed. The pancreas was then separated from the spleen and duodenum, cleared of connective tissue and external fat, weighed, and cut into two sections. Tissue samples were placed in a fixative (mixture of 75 ml water, 25 ml formaldehyde (37%), and 5 ml glacial acetic acid) for 48 hours at room temperature. They were washed three times in phosphate buffered saline (PBS) (pH 7.4), then left in PBS at 4° C overnight. Samples were washed three times in 70% alcohol, placed in cassettes and embedded in paraffin. Two sets of five serial sections (4μm) from each block (4 sets per animal) were cut using an Olympus microtome (CUT 4060; Carsen Group, Markham, ON, Canada) and were mounted on poly-L-lysine coated slides.

Staining. Three slides from each serial section were stained for insulin-BrdU, insulin, insulin-TUNEL. All sections were dewaxed in xylene, dehydrated in petroleum ether, and incubated in 0.3% hydrogen peroxide in methanol for 30 min. Sections were then washed in PBS and incubated in 10% lamb serum in PBS for an additional 30 min. Sections were serially incubated in guinea pig anti-insulin antibody (1:1000) (Dako Diagnostics, Mississauga, ON, Canada) at 37° C for 30 min, biotinylated anti-guinea pig antibody (1:500) (Vector Laboratories, Burlington, ON, Canada), for 1 hr and avidin/biotin horseradish peroxidase complex (ABC-HP) (1:1000) (Vectastain Elite ABC Kit; Vector Laboratories) for 1 hr at room temperature. Samples were then developed in 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (Sigma-Aldrich, Oakville, ON, Canada) for 10 min. Sections were washed several times with PBS between incubations. After DAB development, sections were washed in running tap water, counterstained with hematoxylin (Harris, Sigma-Aldrich) and coverslipped with Permount mounting media (Fisher Scientific , Nepean, Ontario, Canada). The sections double stained for Bromodeoxyuridine (BrdU) and insulin were dewaxed and hydrated as described above and then incubated with Anti-Bromodeoxyuridine monoclonal antibody with nuclease

(Amersham Pharmcia Biotech, Baie d'Urfé, Quebec, Canada) for 30 min at 37° C. Sections were washed with PBS and incubated serially with biotinylated goat anti-mouse antibody (Vector) for 1 hr and ABC-HP (1:1000) (Vector) for 1 hr at room temperature. Slides were washed in PBS between incubations. Sections were developed in DAB solution for 2-3 min. Slides were washed with PBS and incubated with guinea pig anti-insulin antibody (DAKO Diagnostics) for 30 min at 37° C. Next they were serially incubated with biotinylated anti-guinea pig antibody (1:500) (Vector) for 1hr and avidin/biotin alkaline phosphatase complex (Alkaline Phosphatase Standard Vectastain ABC Kit, Vector Laboratories) for 1 hr at room temperature. PBS washes were carried out between incubations. Sections were developed in Dako Fuchsin Substrate-Chromogen System for 10 min (Dako Diagnostics). Slides were then washed in water, counterstained with hematoxylin and coverslipped with Permount. Double staining for insulin and TUNEL was performed as previously described (Lipsett and Finegood 2002, O'Brien *et al.* 2002).

Quantitative morpholometry. β-cell mass was determined via quantitative analysis of the insulin antibody stained slides. An image analysis system consisting of an Olympus light microscope (Model BX40; Carsen Group) attached to a Sony color video camera (Model DXC-950; Sony, Japan) and MetaVue image analysis software (Universal Imaging Corporation, Downingtown, PA) was utilized to estimate the β-cell and non-β-cell area on each slide. A point counting system was used to estimate the ratio of adipose to non-adipose tissue on each slide. The ratio of β-cell to non-β-cell tissue was determined for each animal then multiplied by the pancreatic mass to generate an estimate of total β-cell mass. Ten slides were used to determine the relationship between β-cell number and β-cell area. These correlations were then used to estimate the total number of pancreatic β-cells on each slide. Double-stained slides were then manually analyzed to determinate the total number of BrdU or TUNEL positive β-cells, and combined with the β-cell number data to determine the percentage of β-cell undergoing replicating or apoptosis respectively. Each slide was quantified as either having or not

having morphological evidence of infiltration, focal areas, or ductal areas (see Figure 4-4 for pictures).

Statistics. Data are presented as mean ± standard error unless otherwise noted. Comparisons between groups were performed via one-way ANOVA with either a post hoc Dunet's t-test analysis for comparison to control or a post hoc Tukey analysis for comparison between all groups (JMP IN 3.1.5, The SAS Institute, Cary, NC). Linear correlations were performed in SigmaPlot 7.101 (SPSS inc, Chicago, Il).

Results

Forty young Sprague-Dawley rats were divided into 5 groups. Each group was clamped for 24 hours at one of basal, 15, 20, 25, or 35 mmol plasma glucose (Figure 4-1). Mean glucose levels for each animal were within 10% of the targeted levels. Group mean glucose level were held within 3% of targeted levels (15.2 ± 0.2, 19.8 ± 0.4, 25.3 ± 0.3, 35.0 ± 0.7, $p<0.05$ for all comparisons). With the exception of the first hour, and a couple of hours following the i.p. BrdU injection (t = 18hr), group mean plasma glucose levels differed significantly between all cohorts at all points in time.

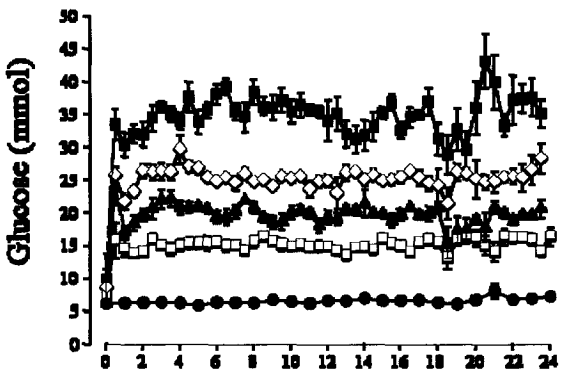

Figure 4- 1 Glucose levels for the basal (black circles), 15 mmol (white squares), 20 mmol (black triangles), 25 mmol (white diamonds), and 35 mmol (black squares) cohorts of animals. N = 8 for each cohort. Data shown as mean ± SEM.

During the first four hours of the hyperglycemic clamp, plasma insulin levels rose to an approximate steady state in each group (Figure 4-2A). These steady state levels displayed a linear relationship to glycemia ($r^2 = 0.33$, $p < 0.05$) (Figure 4-2B). By the end of the study, insulin levels had increased in the 15 mmol and decreased in the 25 and 35 mmol cohorts such that no clear relationship to glycemia remained ($r^2 = 0.04$, p = ns) (Figure 4-2A, B).

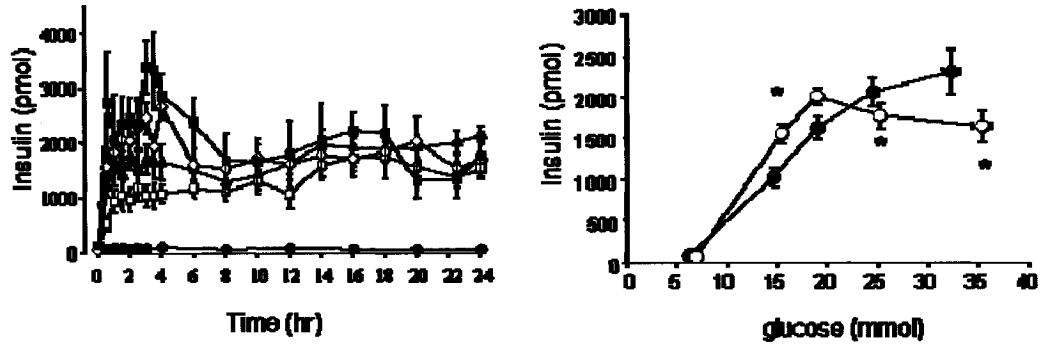

Figure 4- 2 (A) Insulin levels during the 24 hour clamp for the basal (black circles), 15 mmol (white squares), 20 mmol (black triangles), 25 mmol (white diamonds), and 35 mmol (black squares) cohorts of animals. N = 8 for each cohort. Data shown as mean ± SEM. (B) Plasma insulin levels as a function of glycemia during the early (black circles) and late stages (white circles) of the 24 hour clamp. * $p < 0.05$.

During the first four hours of the experiment glucose infusion rates show a strong correlation to blood glucose levels ($r^2 = 0.61$, $p<0.05$) (Figure 4-3A, B). Over the course of the study glucose infusion rates decreased in all groups and tend to converge to a common level.

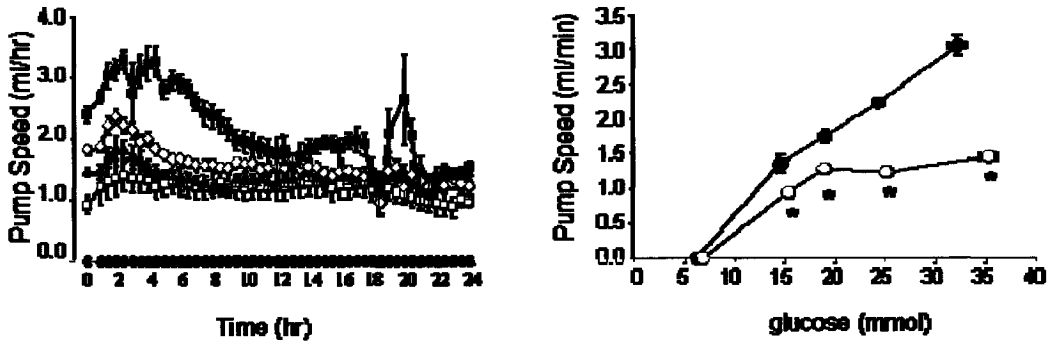

Figure 4- 3 Glucose infusion rates during 24 hour glucose clamp for the basal (black circles), 15 mmol (white squares), 20 mmol (black triangles), 25 mmol (white diamonds), and 35 mmol (black squares) cohorts of animals. N = 8 for each cohort. Data shown as mean ± SEM. (B) Glucose infusion rates as a function of glycemia during the early (black circles) and late stages (white circles) of the 24 hour clamp. * $p < 0.05$.

β-cell mass did not differ between groups (Figure 4-4). Replication rates were decreased in the 15 and 20 mmol groups while death rates were elevated in the 35 mmol cohorts. β-cell replication rates displayed a strong linear relationship to glycemia ($r^2 = 0.98$, $p < 0.05$ for group mean data). It is interesting to note that β-cell replication rates were reduced in animals that displayed significant increases in blood insulin levels while β-cell death rates were elevated in the animals that displayed significant reductions in blood insulin levels.

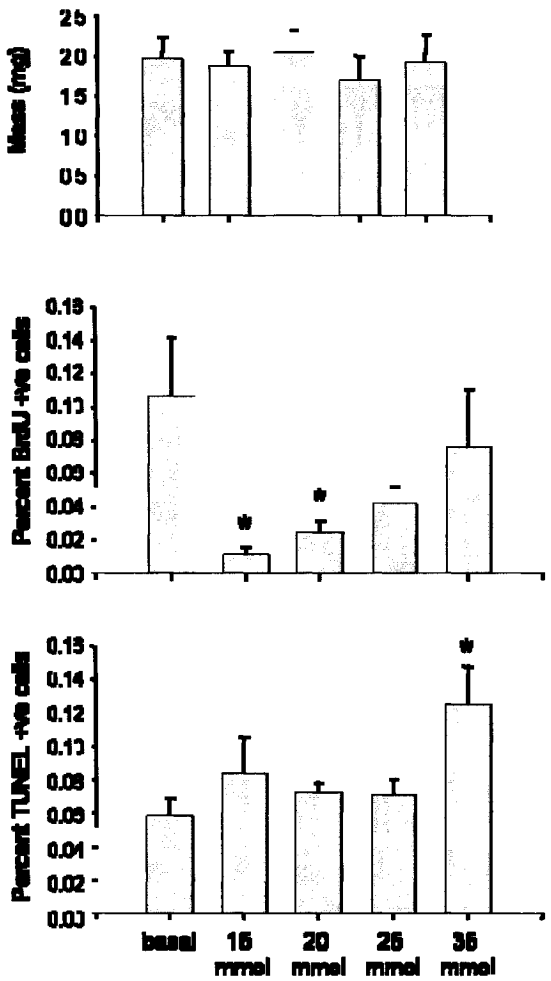

Figure 4- 4 β-cell mass, replication, and death rates in the 5 cohorts of animals. * $p < 0.05$.

Three distinct morphological abnormalities were observed in pancreatic samples from these animals. The first of which, infiltration, is defined as the presence of small replicating cells in the inter-acinar space (Figure 4-5A). The second morphological abnormality, focal areas, consisted of loose, highly replicative, irregular tissue that roughly resembled embryonic pancreatic tissue (Figure 4-5B). The third morphological abnormality, ductal areas, consisted of clusters of ductal-like tissue that displayed both high levels of replicating cells and small clusters of insulin positive cells (Figure 4-5C). Each of these sections contains CD8 positive stained cells (data not shown). The frequency of focal areas did not differ between groups (Figure 4-5 D-F).

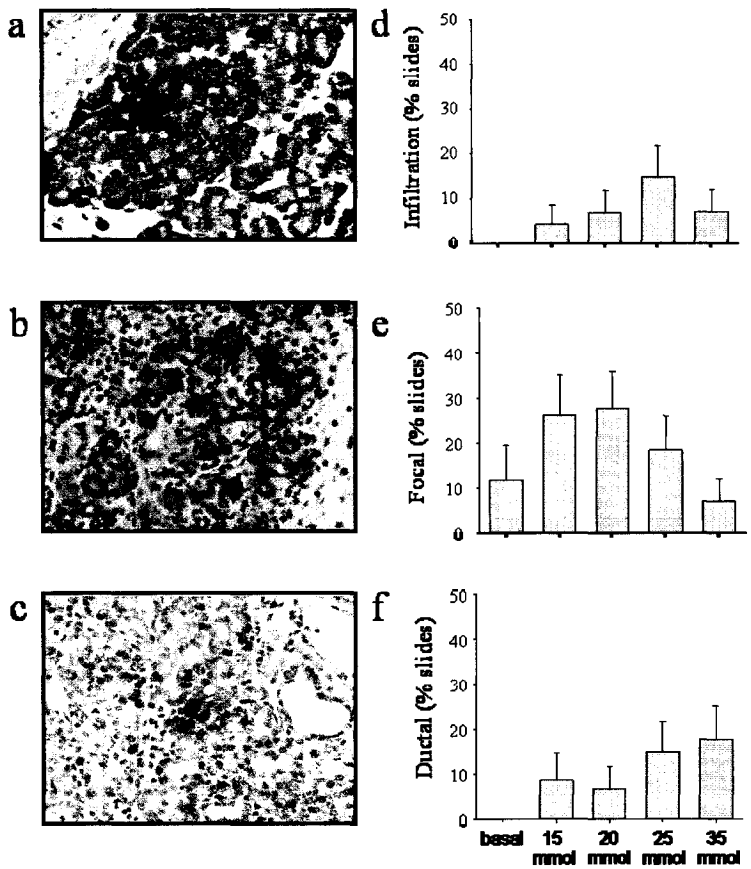

Figure 4- 5 Morphological evidence for neogenesis. (a), (b), and (c) show representative images of infiltration, focal, and ductal areas. (d), (e), and (f) show the relative frequency of these areas in 5 cohorts of animals. * $p < 0.05$

Discussion

Several studies have utilized the chronic glucose infusion protocol to investigate the effects of hyperglycemia on β-cell mass dynamics *in vivo* [28, 36]. The vast majority of these studies have employed a single high rate of glucose infusion. Here we developed a 24 hour glucose clamp protocol to fully quantify the relationship between blood glucose levels and β-cell mass adaptation *in vivo*. Overall, β-cell replication rates displayed a linear relationship to blood glucose levels in all hyperglycemic animals, while apoptosis was only increased in overt hyperglycemia (35 mmol). No clear relationship was observed between morphological indices of neogenesis and blood glucose levels.

Pioneering work by Swenne demonstrated a liner relationship between β-cell replication rates and the concentration of glucose in the medium [18]. Subsequent *in vitro* studies have confirmed this finding in mild to moderate hyperglycemia, but have shown that prolonged exposure to extreme hyperglycemia, or moderate hyperglycemia coupled to hyperlipidemia, led to reduced rates of β-cell replication [20, 19, 12]. Our finding of reduced replication rates in moderate hyperglycemia seems to contradict these previous reports. However, it should be noted that β-cell replication rates displayed a clear linear relationship to glycemia in the 15 to 35 mmol groups. It should also be noted that several other *in vivo* glucose infusion studies have reported reduced rates of β-cell replication after 24 hours of infusion [25, 27, 29, 36] followed by increased β-cell replication rates on later days of study [27, 28]. This may suggest that a transient reduction in β-cell replication rates occurs during the first day of chronic glucose infusion due to stress, diversion of cellular energies to increasing cell size/function, or perhaps due to a transient reduction in another metabolic/hormonal signal such as GLP-1. Together this suggests that our data represents a combination of two responses, 1) a "stress" response that reduced β-cell replication in all hyperglycemic animals and 2) a linear effect of glucose on β-cell replication rates.

In vitro studies have shown moderate hyperglycemia to inhibit β-cell apoptosis while overt hyperglycemia or moderate hyperglycemia coupled to hyperlipidemia has been

shown to increase β-cell death rates (glucoapoptosis, lipoapoptosis) [19, 20]. While it is becoming increasingly clear that hyperglycemia and dyslipidemia contribute significantly to the increased rates of β-cell death that characterize the late stages of type 2 diabetes [12], few studies have investigated the effects of moderate hyperglycemia on β-cell death rates *in vivo*. Bernard *et al.* found β-cell death rates to be reduced following 24 and 48 hours of glucose infusion [25]. However, using a similar experimental protocol and a mathematical modelling approach, we predicted a wave of increased β-cell death during this time period [28]. In this study 24 hours of moderate hyperglycemia did not have any significant effect on the number of TUNEL positive β-cells. However, consistent with our previous model predictions [28], we found overt hyperglycemia to cause increased rates of β-cell apoptosis. Together this suggests that high levels of acute glycemia are required to induce apoptosis *in vivo*.

The role of β-cell neogenesis in β-cell mass adaptation is unclear. *In vitro* studies have shown pancreatic tissue to be plastic and capable of converting from one fully differentiated cell type to another [37, 38, 39]. Also, there is growing morphological evidence to suggest that neogenesis and/or transdifferentiation contribute significantly to expansion of the β-cell mass *in vivo* [28, 29, 25]. However, recent publications by Dor *et al.*, and Bock *et al.* question the relevance of neogenesis or transdifferentiation *in vivo* [40, 41]. Employing a transgenic pulse-chase technique, Dor *et al.* recently found that the vast majority of β-cells formed following a partial pancreatectomy are of β-cell lineage. Bock *et al.* utilized a stereographic technique to show that obese ob/ob mice have the same number of islets as lean ob/ob mice implying that the increased β-cell mass observed in obese ob/ob mice was derived from expansion (hyperplasia and/or hypertrophy) of cells within the islets.

Here we found three distinct morphological abnormalities, infiltration, focal and ductal areas. It is tempting to suggest that these areas represent the de-differentiation of acinar tissue into ductal, then islet tissue. Several lines of evidence support this concept. First, the observation of constant β-cell mass despite reduced β-cell replication or increased apoptosis suggests the occurrence of neogenesis in this study. Second, these

morphological areas (focal and ductal) are similar to areas previously proposed as morphological evidence of neogenesis [29]. Third, acinar and islet cells are derived from common eptithelial stem cells during pancreatic development [42], suggesting that conversion of one fully differentiated cell type to another would require de-differentiation back into a precursor cell. Finally, both islet and acinar carcinomas tend to de-differentiate into a common adenocarcinoma (infiltrating epithelial cells forming glands of various shapes and sizes) as the disease progresses[4243] suggesting that a loss of differentiation of these cells results in a duct-like phenotype. Overall, this suggests that the morphological evidence found here likely represents pancreatic de-differentiation and possibly new β-cell formation.

While it is tempting to suggest that glucose is the driver for this conversion, the frequency of these areas did not differ between groups (due in part to the unexpected observation of ductal areas in one control animal). Also, morphological aberrations were confined to single pancreatic lobules in an otherwise healthy pancreas. It is difficult to discern how a diffuse signal like plasma glucose would affect one lobe but not others. A possible explanation for these observations is that acinar de-differentiation results from a collapsed exocrine duct secondary to reduced food intake during and before the study. Animals were fasted 12 hours before the study and did not receive food during the study. This reduced food intake is associated with reduced acinar activity and reduced exocrine secretion rates. Low levels of exocrine activity may have caused a duct to collapse leading to a back up of digestive enzymes into the acinar intracellular space. Ductal ligation was an early technique for islet isolation while partial duct obstruction has been shown to cause both neogenesis and morphological aberrations similar to those reported here [49]. Overall, this suggests that the morphological areas observed here represent pancreatic re-modeling and possibly new islet formation.

Finally, we observed adaptation of both β-cell function and the rates of glucose uptake between the early and late stages of the 24 hour clamp. These may well represent known effects of glucose on β-cell function and insulin sensitivity [44, 45]. Our observation of increased blood insulin levels in the moderately hyperglycemic groups, despite constant

β-cell mass and blood glucose levels, implies glucose-induced adaptation of β-cell function similar to previous findings [28, 46]. Similarly, the decrease in blood insulin levels observed during the 24 hour clamp is consistent with other studies that have reported toxic effects of extreme hyperglycemia on pancreatic β-cell function [47]. However, it is interesting to note that by the end of the study, all groups displayed roughly similar blood insulin levels. This may suggest that under conditions of chronic hyperglycemia, β-cells adapt to secreting insulin at a maximal sustainable rate, independent of the degree of hyperglycemia. Along a similar vein, our observation of reduced rates of glucose infusion during the course of the study is consistent with the concept of glucose induced insulin resistance [45] and may well imply a proportional relationship between glycemia and insulin resistance. However, the observation of a common glucose infusion rate for all groups at the end of the study may also imply a maximal sustainable rate of glucose uptake, likely equal to the maximal rate of glucose metabolism. This would imply that during the early stages of the clamp, muscle cells can take up more glucose than they metabolize. However, as glycogen stores fill and glycolytic metabolites begin to accumulate, glucose uptake becomes inhibited in a manner similar to that described by Randle's glucose free fatty acid cycle [48].

Overall, we found that animals exposed to 24 hours of moderate hyperglycemia displayed increased β-cell function and decreased β-cell replication rates while animals exposed to 24 hours of overt hyperglycemia displayed reduced β-cell function and increased rates of β-cell apoptosis. Our observation of a strong linear relationship between glycemia and β-cell replication rates in all hyperglycemic animals suggests that glucose does regulate β-cell replication rates *in vivo*. However, the fact that β-cell replication rates in the non-infused animals did not fit on this line may imply the existence of a second regulatory signal/process that is active in glucose infused, but not control animals. Our results support the concept of gluco-apoptosis at extreme hyperglycemia, but did not reveal any relationship between moderate hyperglycemia and β-cell apoptosis *in vivo*. Finally, this study provides further morphological and quantitative evidence to support the concept of glucose induced neogenesis/transdifferentiation.

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DISCUSSION

While it is becoming increasingly clear that β-cell mass is elevated in obesity and reduced in type 2 diabetes, the mechanisms responsible for these adaptations remain unclear [1, 2]. Further it is unclear if aberrant β-cell mass dynamics play a primary or secondary role in the pathogenesis of type 2 diabetes. It can be argued that this uncertainty arises naturally from a lack integrative and dynamic analysis. This thesis focused on the development and application of novel methodologies to investigate the relative dynamics of, and mechanisms governing, β-cell mass, insulin sensitivity, and β-cell function during the development of obesity and type 2 diabetes. First, a mathematical model of coupled β-cell mass, insulin and glucose (βIG) dynamics was developed and utilized to investigate the mechanistic links connecting insulin sensitivity, β-cell mass and β-cell function. Simulation and bifurcation analysis of this model (Chapter 1) suggested that glucose is a reasonable signal connecting insulin sensitivity to β-cell mass dynamics and that type 2 diabetes occurs when insulin sensitivity decreases faster than the β-cell mass expands. To test these hypotheses we; 1) validated the βIG model as an indirect method for estimating insulin sensitivity and β-cell function in small rodents (Chapter 2), 2) utilized the βIG model to estimate the dynamics of insulin sensitivity, β-cell function and β-cell mass during the development of diabetes in ZDF rats (Chapter 3) and 3) employed a 24 hour hyperglycemic clamp protocol to quantify the relationship between glycemia and β-cell mass dynamics *in vivo* (Chapter 4). Key findings from these studies include quantitative support for β-cell neogenesis during chronic glucose infusion (Chapter 2), the observation that the early stages of type 2 diabetes in ZDF rats is characterized by abnormalities in both insulin sensitivity and β-cell mass expansion (Chapter 3) and the observation that, during a 24 hour hyperglycemic clamp, β-cell mass adaptation *in vivo* shows no clear relationship glycemia (Chapter 4).

Physiological Insights

This thesis focused on two primary questions; 1) is glucose the signal connecting insulin sensitivity to β-cell mass adaptation *in vivo*, and 2) what are the relative contributions of insulin resistance, insulin secretory defects and β-cell mass dynamics to the pathogenesis of type 2 diabetes. Although results from Chapters 1 and 2 provide indirect evidence to suggest that glucose may regulate β-cell mass dynamics *in vivo*, experimental data from Chapters 3 and 4 do suggest that glucose is not a primary driver of β-cell mass dynamics *in vivo*. Simulation results from Chapter 1, combined with experimental data from Chapter 3 suggest that the etiology of type 2 diabetes in ZDF rats lies in two distinct defects, slow β-cell mass adaptation and rapid development of insulin resistance.

Regulation of β-cell mass dynamics *in vivo*. Several studies have indicated a feedback loop connecting peripheral insulin action to pancreatic β-cell adaptation [34, 5], however, the signals connecting these metabolic processes remain highly contested. Glucose is a logical signal as it is affected by insulin action and easily detected by pancreatic β-cells. This hypothesis is supported largely by *in vitro* studies that demonstrated nonlinear effects of glucose on rates of β-cell replication and death [6, 7]. The effects of glucose on β-cell mass adaptation *in vivo* are less clear. Short term (1-2 days) glucose infusion generates both hyperglycemia and β-cell mass adaptation [8]. However, prolonged glucose infusion (3-6 days) is associated with continued β-cell mass adaptation despite the re-establishment of normoglycemia [9]. β-cell mass adaptation in the absence of hyperglycemia has also been observed in other experimental paradigms and transgenic animal models [10]. Results from this thesis are similarly mixed and difficult to interpret.

βIG model simulations from Chapter 1 provide some support for glucose as a signal. The β-cell mass equation in Chapter 1 was based on the known effects of glucose on β-cell replication and death *in vitro*. By incorporating this equation into a minimal mathematical representation of the glucose regulatory system, we were able to reproduce both the normal β-cell adaptation to obesity and the "Starling" curve insulin dynamics that characterize type 2 diabetes. While this does not prove that glucose is the signal

connecting insulin resistance to β-cell mass dynamics, it does show that the known effects of glucose on β-cell mass adaptation *in vitro* are sufficient to explain the complex β-cell mass dynamics observed in these metabolic states.

Data from Chapter 2 provided mixed results on the role of glucose in regulating β-cell mass dynamics *in vivo*. Both plasma glucose levels and β-cell replication rates displayed nonlinear responses to chronic glucose infusion. Interestingly, plotting β-cell replication rates against blood glucose concentrations reveals a bell curve similar to that reported *in vitro* [11]. However, replication rates remained significantly elevated during the last couple of days of glucose infusion when glucose levels did not significantly differ from saline-infused animals. It should also be noted that βIG model analysis of this data predicted two waves of net neogenesis, one occurring concurrent with overt hyperglycemia while the other occurred during a period of sustained normoglycemia.

Data reported in Chapter 3 suggests that glucose is not the signal connecting insulin resistance to β-cell mass adaptation. Between 6 and 12 weeks of age, both the male ZDF and low fat fed female ZDF rat displayed exacerbation of insulin resistance, transient increases in β-cell replication rates and transient expansion of the β-cell mass. However, blood glucose levels remained largely unchanged during this period and did not display any relationship to either replication rates or β-cell mass. In addition, while blood glucose levels differed significantly between low and high fat fed female ZDF rats neither β-cell mass nor β-cell replication rates differed between these groups at any point in time. This observation is consistent with other studies that have shown significant β-cell mass adaptation in the absence of detectible hyperglycemia [10, 12] but challenges the well accepted theory of β-cell glucotoxicity [13]. Overall, this data suggests that glucose is not a dominant regulator of β-cell mass dynamics during the development of obesity or the pathogenesis of type 2 diabetes.

Direct manipulation of plasma glucose levels in Chapter 4 also provided little evidence to suggest that glucose is a signal for β-cell mass adaptation *in vivo*. In stark contrast to the

bell shaped relationship between β-cell replication rates and glucose levels reported *in vitro* [7], the frequency of BrdU positive β-cells displayed a "U" shaped relationship to glycemia *in vivo*. However, it should be noted that if the control group is excluded, β-cell replication rates displayed a linear relationship to glycemia. Suggesting that the overall "U" shaped relationship between glycemia and replication rates is the result of two signals, a "stress" signal that lowers replication rates, and a glucose signal that increases replication rates. Also in contrast to previously published *in vitro* data, the frequency of TUNEL positive β-cells was not inhibited by moderate hyperglycemia *in vivo*. Although we reported morphological evidence of neogenesis, neither the size nor frequency of appearance of these areas displayed any clear relationship to blood glucose levels. Overall this data provides little if any support for the concept that moderate hyperglycemia acts as a signal for expansion of the β-cell mass.

Overall, our observations of β-cell mass adaptation in response to glucose infusion (Chapters 2 and 4) clearly indicate that glucose has direct or indirect effects on β-cell mass dynamics *in vivo*. Also the correlations observed between glycemia and β-cell replication in these Chapters suggests a possible direct causal relationship. However, our observations of β-cell mass adaptation in the absence of hyperglycemia (Chapter 2, 3) suggest that glucose is not the only regulator of β-cell mass *in vivo*. Clearly, further study is required to identify the other regulatory signals and to determine their relative importance to obesity and type 2 diabetes.

Insulin sensitivity, β-cell function and β-cell mass in type 2 diabetes. While type 2 diabetes has long been characterized by insulin resistance, insulin secretory defects and reduced β-cell mass, the relative contributions of these defects to the pathogenesis of this disease remains unclear. There is growing evidence to suggest that the hyperglycemia and dyslipidemia of type 2 diabetes contributes significantly to disease progression[13, 14, 15]. This diabetic milieu has been shown to exacerbate insulin resistance, cause insulin secretory defects and lead to increased rates of apoptosis. These observations have focused the question of etiology on the events responsible for the initiation of

hyperglycemia and dyslipidemia. However, few studies have examined this time period. Most of these studies concentrated on a single aspect of metabolic regulation at a single point in time. As a result, relatively little is known about the dynamics of insulin sensitivity, β-cell function and β-cell mass during the initiation of type 2 diabetes.

In this thesis, two distinct approaches were used to address this issue. First, a mathematical modeling approach was employed to incorporate data from diverse studies into an integrative and dynamic hypothesis about the pathogenesis of type 2 diabetes. Second, the βIG model was utilized as a data analysis tool to quantify the full dynamics of insulin sensitivity, β-cell function and β-cell mass during the development of obesity in two animal models of type 2 diabetes and their obese controls.

Bifurcation and simulation analysis of the βIG model in Chapter 1 characterized type 2 diabetes as a dynamic process where insulin resistance decreases faster than the maximal rate of β-cell mass adaptation. This may be due to excessive insulin resistance, slow β-cell adaptation or both. Once glucose levels become high enough, they become toxic and initiate a cascade of progressive β-cell defects. To test this hypothesis we quantified insulin sensitivity, β-cell function, β-cell mass, β-cell replication and net-neogenesis in two common animal models of type 2 diabetes (Chapter 3).

The findings of Chapter 3 supported our dynamic hyperglycemia hypothesis. Hyperglycemia was not preceded by a marked reduction in blood insulin levels or a wave of β-cell apoptosis. Initiation of hyperglycemia was characterized by rapid reductions in insulin sensitivity and impaired expansion of β-cell mass. Insulin secretory defects only became apparent after overt hyperglycemia was established. This was the first study to show that insulin sensitivity decreases more rapidly in ZDF than ZF rats. It was also the first study to show β-cell mass dynamics to be abnormal during the initiation of hyperglycemia. Pick *et al.* had shown β-cell mass to be normal in pre-diabetic ZDF rats and abnormal in fully hyperglycemic ZDF rats [16]. However, it was unclear if this relative reduction in β-cell mass was due to slow expansion (primary defect) or glucotoxicity

induced apoptosis (secondary defect). Also, this study was the first to show that insulin secretory defects do not contribute to the initiation of hyperglycemia in these animals. Several other studies have shown β-cell function to be normal in pre-diabetic ZDF rats and abnormal in fully diabetic ZDF rats. However, similar to β-cell mass, it was unclear if these insulin secretory defects played a primary role in the initiation of hyperglycemia or were secondary to gluco-lipotoxicity.

The mechanism(s) responsible for excessive insulin resistance and impaired β-cell mass adaptation in diabetes prone ZDF rats remains unclear. The observation of similar body weight and caloric intake between pre-diabetic and control animals suggests that the excessive insulin resistance in these animals may be due to abnormal lipid partitioning rather than whole body lipid accumulation. Accumulation of lipid stores in visceral adipose, or non-adipose tissue (liver and muscle) has been shown to cause insulin resistance[17, 18]. This suggests that abnormalities in lipid uptake/metabolism can lead to a detrimental distribution of lipid stores and excessive insulin resistance independent of whole body adiposity. Slow β-cell mass adaptation may represent a second distinct defect; however, there is some data to suggest that triglyceride accumulation in pancreatic β-cells can impair β-cell mass adaptation [14]. Further research is required to discern the mechanisms responsible for these observations.

Overall this study provides evidence to suggest that both excessive insulin resistance and insufficient β-cell mass adaptation play primary roles in the pathogenesis of type 2 diabetes while insulin secretory defects are a secondary event in disease progression. However, it is unclear if these represent two distinct defects or if these events occur secondary to a common primary defect (such as abnormal lipid partitioning). This issue can be partially addressed via quantification of the dynamics of non-adipose lipid accumulation during the development of obesity in these animal models.

Methodological Advances

Recent methodological advances in genetic and molecular biology have revolutionized experimental physiology and drug discovery. Developments such as transgenic knock out/in technology, anti-sense mRNA, gene chips, and RT-PCR have allowed researchers to perform previously impossible experiments and gather more data, faster, and cheaper than ever before. While these methodologies have vastly improved our ability to manipulate specific molecular targets and quantify the integrative and dynamic response to these perturbations at a cellular level, quantifying the indices of whole body metabolic regulation (eg. insulin sensitivity) remains difficult, and time consuming.

The study of β-cell mass dynamics is hindered primarily by limitations of histological analysis. The need to quantify β-cell mass and replication rates using histological methods prevents longitudinal analysis. Cross-sectional analysis is time consuming, expensive, and noisy (due in part to inter-animal variation). Further, there is no accepted means of quantifying the rates or magnitudes of β-cell apoptosis or neogenesis. While histological markers can be used to suggest that one cohort expresses higher/lower rates than the other, the relative contributions of these processes to overall β-cell mass dynamics remain elusive. Finally, the slow nature of β-cell mass adaptation has made it difficult to quantify the relationship between possible regulatory signals and components of β-cell mass adaptation. Manipulation of a metabolic signal, such as blood glucose or free fatty acid levels, generally leads to metabolic adaptations of muscle, liver and adipose tissue that make it difficult to maintain targeted signal levels over the required time period.

In addition to the difficulties in measuring β-cell mass dynamics, discerning the relative importance of these dynamics to the overall pathogenesis of type 2 diabetes requires quantification of the dynamics of other metabolic indices. Most notable of these indices are insulin sensitivity and β-cell function. Both of these indices are also difficult to measure in small laboratory animals.

During the course of this thesis research two novel approaches were utilized to investigate the mechanisms governing β-cell mass dynamics *in vivo*, as well as the relative contributions of aberrant β-cell mass dynamics in the pathogenesis of type 2 diabetes in the *fa/fa* rat model. The first methodology was the application of bifurcation theory to the study of type 2 diabetes. Since present techniques only allow for quantification of a limited number of indices at one or two points in time, it is important to be able to incorporate diverse data from different studies into an integrated understanding of metabolic regulation and progression to hyperglycemia. Mathematical modeling provides a powerful means of investigating the interactions of different components of physiological regulation as well as determining the effect of aberrations in individual feedback loops on whole system behaviour. The second methodological advance was the development of a technique to allow for indirect estimation of insulin sensitivity, β-cell function, and net-neogenesis in small animal models. This will make it far more practical to gather integrative and dynamic data during metabolic adaptations such as the development of obesity or type 2 diabetes.

Indirect assessment of metabolic indices in small laboratory animals. Insulin sensitivity and β-cell function are two metabolic indices central to glycemic control. However, direct experimental assessment of these indices is complex, time consuming and expensive. In small laboratory animals, insulin sensitivity has traditionally been quantified using the hyperinsulinemic clamp. Multiple techniques have been used to quantify β-cell function in this population. These techniques include the hyperglycemic clamp (*in vivo*), perfused pancreas (*ex vivo*), and isolated islets (*in vitro*). Each of these techniques is complex, and does not lend itself to dynamic or integrative study, and generate differing estimates of β-cell function [5].

Approximately two decades ago, development of the minimal-model and HOMA models provided practical means of estimating insulin sensitivity and β-cell function in humans or large laboratory animals [19, 20]. The value of these techniques is apparent by the observation that these methodologies have been used in hundreds of clinical studies

largely replacing the hyperinsulinemic and hyperglycemic clamps as a method for measuring insulin sensitivity and β-cell function in humans [21, 22]. However, due to species differences (minimal-model) and model complexity (HOMA) these techniques have yet to be adopted for rats or mice.

Chapter 2 describes the development and validation of the βIG model into a data analysis tool for estimating insulin sensitivity and β-cell function from plasma glucose and insulin levels in rats. By utilizing steady state instead of a response-to-stimulus protocol it was possible to both simplify the methodology and sidestep the species differences that have prevented application of the minimal-model to small laboratory animals [23]. This approach required that all non-identified parameter values be assigned population mean values. Unlike the HOMA model that would require the experimental determination of over a dozen relatively obscure physiological parameters, the βIG model only required experimental determination of four relatively common physiological indices. These parameters were easily derived from previously published experimental data. In addition the βIG model was utilized to estimate net-neogenesis (neogenesis – death) from β-cell mass and replication rate data.

In Chapter 2, the βIG model was applied to chronic glucose infusion data. The ability of the βIG model to accurately estimate the complex dynamics of insulin sensitivity, β-cell function (defined as the ratio of insulin secretory capacity and insulin clearance rates) and neogenesis during chronic glucose infusion provides validation for this methodology in small animal models. In addition, the ability to estimate the dynamics of each of these variables in a single study highlights the utility of this approach

Application of bifurcation theory to metabolic regulation. Bifurcation theory is a branch of mathematics that examines the behaviour of complex nonlinear systems. The term bifurcation describes a change from one qualitative behaviour to another. The number of qualitative behaviours that a system is capable of displaying arises naturally from the nonlinear interactions of its components. Combining the observations that physiological

systems generally consist of complex nonlinear interactions and that pathological states are qualitatively distinct from normal physiology, Mackie and Glass developed the concept of dynamical diseases [24]. This theory suggests that a pathological change in a single component of a complex physiological network can result in a full qualitative change in the behaviour of that system. Unlike traditional modeling techniques that tended to incorporate linear assumptions or restrict analysis to approximately linear regions of behaviour, Mackie and Glass showed that analysis of the intrinsic nonlinearities of physiological systems could help explain complex behaviours such as changes in regulation, hysteresis, and multiple distinct etiologies for a single pathological condition.

One of the goals of Chapter 1 was to bring the tools of bifurcation theory to the study of type 2 diabetes. The general approach was to: 1) develop a minimal mathematical representation of the glucoregulatory system that incorporated the nonlinear interactions of the represented components, 2) identify all possible qualitative sets of behaviour that this model was capable of displaying, and 3) identify all possible mechanisms for switching from one behaviour to another. More specifically, the goal was to develop a minimal representation of the mechanistic links between peripheral insulin resistance and β-cell adaptation to investigate all possible mechanisms of generating hyperglycemia in this model.

Overall, we found three qualitative pathways to hyperglycemia. These pathways provided dynamics similar to those observed in maturity onset diabetes of the young (MODY), type 1 diabetes and type 2 diabetes; the latter two pathways being bifurcation processes. This is the first description of type 2 diabetes as a dynamical disease. There are two primary implications of this analysis. First, these results suggest that type 2 diabetes is a heterogeneous disease; several distinct primary defects are capable of generating the qualitative set of behaviour that defines type 2 diabetes. Second, this analysis should provide new impetus to quantify the dynamics of metabolic indices. While much of the experimental literature is focused on magnitudes of metabolic indices at fixed points in time, this analysis suggests that the rate of development of insulin

resistance and the rate of β-cell mass adaptation are the primary drivers of long term metabolic control.

Looking to the future, lipid metabolism should be incorporated into the next generation of the βIG model. There is increasing evidence to suggest that the root cause of diabetes lies in abnormal lipid metabolism or partitioning [14, 15]. Insulin resistance is strongly correlated to total adiposity, central adiposity, and triglyceride accumulation in muscle and liver tissue [17, 18]. Also, acute exposure of pancreatic β-cells to hyperlipidemia has been shown to potentiate glucose-induced insulin secretion while chronic increases in β-cell triglyceride stores have been associated with insulin secretory defects and increased rates of apoptosis [14, 18]. This has led some to suggest that primary abnormalities in lipid buffering at the adipocyte, or lipid metabolism in non-adipose tissue, leads to abnormal distributions of lipid stores and eventually type 2 diabetes [15]. Incorporation of the regulation of lipid buffering, distribution, and metabolism into the βIG model would allow for a more mechanistic analysis of the causes of insulin resistance as well as the mechanisms of β-cell adaptation and failure.

SUMMARY

Dr Dennis McGarry described the question of what causes type 2 diabetes as "one of the most frequently asked and least satisfactorily answered in the history of diabetes research" [17]. This is due in large part to the complex and dynamic nature of type 2 diabetes. Later in the same Banting lecture McGarry highlighted this point by suggesting that, over the course of the disease, type 2 diabetes is characterized by progressive defects in nearly every aspect of metabolic regulation. This complexity has hindered the full experimental characterization of this disease, and has placed increasing emphasis on the analysis of the early stages of its development. It has been widely postulated that a limited number of primary defects initiate a diabetogenic milieu that causes additional progressive metabolic defects. Identifying these targets will require integrative and dynamic analysis. Success is most likely to arise from a top down approach that first

identifies the primary defects at the level of whole body metabolic regulation followed by directed "drilling down" to mechanism.

Utilizing such an approach we were able to generate several novel insights. Chapter 1 challenged the notion that hyperglycemia is initiated when β-cell defects cause blood insulin levels to fall. We proposed that hyperglycemia arises while insulin levels are still increasing; that long term glycemic regulation is a race between the rate of development of insulin resistance and the rate of β-cell mass adaptation. Chapter 2 provided the strongest evidence yet showing that neogenesis contributes significantly to β-cell mass adaptation in adult animals. The strength of our prediction lies in the fact that our calculation is based entirely on well established histological measurements, β-cell mass and β-cell proliferation rates. Chapter 3 utilized a powerful new methodology to quantify the full dynamics of the primary metabolic indices of metabolic regulation during the initiation and exacerbation of hyperglycemia in ZDF rats. This data supported our claim from Chapter 1 that the initiation of hyperglycemia occurs while insulin levels are rising and is secondary to a mismatch between the rates of development of insulin resistance and β-cell mass expansion. Data from this chapter also clearly show insulin secretory defects occur secondary to the development of overt hyperglycemia. Finally Chapter 4 quantified, for the first time, the full dose response relationship between glucose and β-cell mass adaptation *in vivo*. While these results did not agree with previous *in vitro* reports, the observation of a clear linear relationship between glycemia and β-cell replication rates suggests that glucose may be a primary driver of β-cell mass dynamics *in vivo*.

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