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Andersson, Lotta

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Eva Lotta Andersson

DOCTORAL DISSERTATION

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Faculty opponent

Professor Pierre Maechler

Department of Cellular Physiology and Metabolism

Faculty of Medicine, Université de Genève, Switzerland

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Stimulus-Secretion Coupling in Endocrine Cell Models

Abstract

Detailed understanding of biological systems governing specific mechanisms and pathways is essential in the development of novel disease therapies. Stimulus-secretion coupling in hormone secreting cells is a complex system of pathways that link activation of cellular processes by i.e. nutrients to the release of hormone. Stimulus-secretion coupling in the insulin secreting beta-cell is intensely researched to improve our understanding of type 2 diabetes (T2D), a perpetually growing global pandemic. Such research requires availability of model systems that are metabolically and functionally faithful to the cell type they represent.

In my research I have characterized, evaluated and applied cell models for metabolic research in endocrine cells. In studies I and II cellular function in response to nutrient stimuli in human and murine beta cell models and isolated islets was evaluated. In these studies I investigated functional aspects such as insulin secretion as well as metabolic changes such as changes in intracellular metabolite levels, oxygen consumption rates and energy production. I found that the response was qualitatively similar in human and rat beta cell models. The same was found when comparing a clonal rat beta cell model to isolated rat islets. Hence, I concluded that the similarities outweigh the differences and as such the in vitro models investigated lend themselves useful in metabolic studies, but with the recommend use of primary material for confirmation of key findings.

Stimulus-secretion coupling has been widely studied in the beta cell, providing extensive knowledge on the mechanisms governing insulin secretion elicited by various nutrients. In study III and IV, I used established alpha, beta and L-cell models to investigate similarities and differences in stimulus-secretion coupling in different endocrine cell types. In study III, an important difference in mitochondrial shuttles was found between alpha and beta cells, highlighting the malate-aspartate shuttle to be critical for glucagon secretion. These findings were subsequently verified in mouse islets. In study IV, a striking difference in the activity of glutamate dehydrogenase was found between L and beta cells.

Accumulation of glycogen in the beta cell has been associated with T2D. In study V, I investigated the potential role of glycogen metabolism in beta cells and its potential role in regulation of insulin secretion. This study revealed glycogen metabolism to be active and accumulation of glycogen to occurs in response to elevated glucose levels both in a clonal cell line and human islets. Moreover, perturbation of glycogen metabolism was shown to decrease insulin secretion in vitro.

Stimulus-secretion coupling is highly complex as is the pathogenesis of T2D. These studies highlight how well-characterized metabolic models may be used to further the understanding of stimulus secretion coupling in endocrine cells.

Key words: Stimulues-secretion coupling, beta-cells, alpha-cells, L-cells, malate-aspartate shuttle, GDH, glycogen

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Eva Lotta Andersson

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To my family, near and far

"Any sufficiently advanced technology is indistinguishable from magic."
Arthur C. Clarke

Content

List of Publications

Studies included in the thesis

I. Andersson LE, Valtat B, Bagge A, Sharoyko VV, Nicholls DG, Ravassard P, Scharfmann R, Spégel P, Mulder H (2015). Characterization of Stimulus-Secretion Coupling in the Human Pancreatic EndoC-βH1 Beta Cell Line. PLoS ONE 10(3): e0120879

II. Spégel P*, Andersson LE*, Storm P, Sharoyko VV, Göhring I, Rosengren AH, Mulder H. (2015). Unique and Shared Metabolic Regulation in Clonal β-Cells and Primary Islets Derived From Rat Revealed by Metabolomics Analysis. Endocrinology 156(6): 1995-2005

III. Stamenkovic JA, Andersson LE, Adriaenssens AE, Bagge A, Sharoyko VV, Gribble F, Reimann F, Wollheim CB, Mulder H, Spégel P. (2015) Inhibition of the malate-aspartate shuttle in mouse pancreatic islets abolishes glucagon secretion without affecting insulin secretion. Biochem J. 486: 49-63

IV. Andersson LE, Shcherbina L, Wollheim CB, Mulder H, Wierup N, Spégel P. Amino acid elicited secretion of glucagon-like peptide 1 (GLP-1) from colonic L-cells is governed by a constitutively active glutamate dehydrogenase. Manuscript

V. Andersson LE, Nicholas L, Sun J, Sharoyko VV, Mulder H, Spégel P. Glycogen metabolism in the glucose sensing and supply driven β-cell. Manuscript

Studies not included in the thesis

I. Tang Y, Axelsson AS, Spégel P, Andersson LE, Mulder H, Groop LC, Renström E, and Rosengren AH. (2014) Genotype-based treatment of type 2 diabetes with an alpha2A-adrenergic receptor antagonist. Sci Transl Med 6, 257ra139

II. Göhring I, Sharoyko VV, Malmgren S, Andersson LE, Spégel P, Nicholls DG, and Mulder H. (2014) Chronic high glucose and pyruvate levels differentially affect mitochondrial bioenergetics and fuel-stimulated insulin secretion from clonal INS-1 832/13 cells. J Biol Chem 289, 3786-3798

III. Spégel P, Sharoyko VV, Göhring I, Danielsson AP, Malmgren S, Nagorny CL, Andersson LE, Koeck T, Sharp GW, Straub SG, Wollheim CB, and Mulder H. (2013) Time-resolved metabolomics analysis of β-cells implicates the pentose phosphate pathway in the control of insulin release. Biochem J. 289, 3786-3798

IV. Malmgren S, Spégel P, Danielsson AP, Nagorny CL, Andersson LE, Nitert MD, Ridderstråle M, Mulder H, and Ling C. (2013) Coordinate changes in histone modifications, mRNA levels, and metabolite profiles in clonal INS-1 832/13 beta-cells accompany functional adaptations to lipotoxicity. J Biol Chem 288, 11973-11987

Abbreviations

AAT	Aspartate aminotransferase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
DPP-4	Dipeptidyl peptidase-4
ETC	Electron transport chain
FADH	Flavin adenine dinucleotide
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone
GC	Gas chromatography
GDH	Glutamate dehydrogenase
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon like peptide 1
GLUT	Glucose transporter
GSIS	Glucose-stimulated insulin secretion
GTP	Guanosine-5'-triphosphate
GYS	Glycogen synthase
K_{ATP}-channels	ATP-sensitive potassium channels
LADA	Latent autoimmune diabetes in adults
MDH	Malate dehydrogenase
MODY	Maturity-onset diabetes in the young
MS	Mass spectrometry

NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OCR	Oxygen consumption rate
OPLS-DA	Orthogonal projections to latent structures - discriminant analysis
OxPhos	Oxidative phosphorylation
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
PVDF	Polyvinylidene fluoride
PYGB	Phosphorylase, glycogen; brain
qPCR	Quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
SGLT2	Sodium-glucose co-transporter type 2
siRNA	Short interfering RNA
SIRT4	Sirtuin 4
SUS	Shared and unique structures
SV40LT	Simian vacuolating virus 40 large T antigen
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCA-cycle	Tricarboxylic acid-cycle

Introduction

The importance of hormones in metabolic control

Documents from the ancient world describe what is believed to be diabetes as early as 1500 BCE by the ancient Egyptians. The ailment was most often identified by the sweet urine of the individuals suffering from it, a result of loss of glucose control. The lives of those suffering from this disease were described as short, disgusting and painful, showing the importance of glucose control and homeostasis. The endocrine system serves all living organisms as the means of maintaining metabolic control and consists of a number of glands secreting signaling molecules, hormones, into the circulatory system. Hormones are a means of communication between organs. Hormonal targets are generally distant to the secretion site, but also include signaling molecules that act internally (autocrine) and on cells in close vicinity (paracrine). Hormones are chemically diverse compounds, which can be both water and lipid soluble and are key regulators for a multitude of physiological functions, such as metabolism, growth/development, stress, sensory perception, tissue function and behavior. The endocrine system encompasses many different tissues and glands, but the organ central to glucose homeostasis is the pancreas.

The role of the pancreas and Islets of Langerhans

The pancreas serves two roles in metabolic control; an endocrine role to maintain glucose homeostasis and an exocrine role to secrete enzymes for food digestion into the intestinal tract. Glucose homeostasis is maintained by myriad signaling cascades and metabolic pathways, involving different organs, cell types, hormones and cofactors. Within the pancreas are structures called islets of Langerhans, first described by Paul Langerhans in 1869, which are vascularized high density cell clusters, often referred to as micro-organs, whose architecture varies between species (1). Islets are complex cellular conglomerates which house key glucose regulating cell types; glucagon-secreting alpha and insulin-secreting beta cells (up to 90% of its cellular mass), along with a number of other cells types: somatostatin-secreting delta cells, pancreas polypeptide-producing PP cells,

Ghrelin-producing epsilon cells and vascular cells (2-4). The internal signaling within the islet is crucial for its proper function but is not yet fully understood.

Hormone secretion and its role in postprandial glucose elimination

In healthy individuals, the physiological response to the rise in postprandial plasma glucose levels is release of the anabolic hormone insulin by pancreatic beta cells. Insulin signals to peripheral tissues - skeletal muscle, adipose tissue and liver - to initiate anabolic processes, triggering the cells to utilize the glucose present in the blood, thereby lowering circulating plasma glucose levels. Beta cells are considered glucose-sensing; secretion of insulin is in direct relation to the concentration of glucose they are exposed to (5). After the initial direct response, insulin secretion is augmented by a number of supplementing pathways and molecules (6-8). Some signals are autocrine, others secreted as a result of the meal directly, or a change in plasma blood glucose levels. Certain molecules may serve to signal in dual fashion, both as hormone and via the nervous system (9).

Glucose is taken up by beta cells through glucose transporters, GLUT1/3 in humans and GLUT2 in rodents (10,11), although some recent studies implicate a role for GLUT2 also in human islets (12). Glucose passes into the cells in proportion to the external glucose concentration. Upon entry, glucose is phosphorylated to yield glucose-6-phosphate by hexokinase IV (glucokinase). This hexokinase is glucose-specific, has a low affinity for glucose and is not inhibited by its product. Glucose-6-phosphate is the starting substrate for glycolysis, of which pyruvate is the final product, serving as fuel for mitochondrial metabolism. In the mitochondria, pyruvate is fully oxidized in the tricarboxylic acid (TCA)-cycle, yielding reducing power for oxidative phosphorylation resulting in the production of ATP (13), which leads to an increase in the ATP to ADP ratio within the cell. ATP-sensitive potassium channels in the cell membrane close in response to the increasing ATP levels thus preventing potassium flow, which causes depolarization of the plasma membrane. The depolarization, or change in electrical gradient of the cell, triggers the opening of voltage-dependent calcium channels, allowing bivalent calcium to enter the cell. This process, which is generally referred to as the triggering pathway, ultimately results in the release of insulin (14).

A second, less well-characterized pathway, the amplifying pathway, is attributed to the sustained long term biphasic nature of insulin secretion (14-16). The amplifying pathway is stimulated concentration-dependently by glucose; however, it is independent of the ATP-sensitive potassium channels (6,16), and is not active until the calcium concentration in the cell has increased, rendering mitochondrial metabolism essential for glucose-stimulated insulin secretion (GSIS) (17). It has

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been proposed that messenger molecules from various metabolic processes, such as GTP, ATP, cAMP, NADPH, glutamate, malonyl- and long chain acyl-coenzyme A may be governing regulatory factors of this pathway (8,18-24). These potentiating factors lack the ability to elicit insulin secretion on their own but may be responsible for up to 70% of the total insulin release from the cells (25).

The counter regulatory hormone glucagon and its secretion

When plasma glucose levels drop, either due to the action of insulin or due to starvation, insulin secretion cease and the counter-regulatory catabolic hormone glucagon is released by the pancreatic alpha cells. Glucagon serves to mobilize glucose and signals primarily to the liver to initiate the breakdown of glycogen, glycogenolysis, and production of glucose from non-carbohydrate substrates, gluconeogenesis, resulting in the production and release of glucose into the blood stream to counteract the deleterious effects of hypoglycemia (26,27). The trigger of glucagon secretion is less well established but has been suggested to be under paracrine control or directly regulated by levels of glucose (26-28), insulin (29) and incretins (30). This creates a feedback system to ensure healthy glucose homeostasis. Both insulin and glucagon secretion are impacted by incretins from intestinal enteroendocrine cells, glucagon like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), primarily released from enteroendocrine L- and K-cells, respectively (31-34).

Alpha cells are electrically excitable (35,36) and, as in beta cells, this activity is coupled to hormone secretion. There is growing evidence that intrinsic and extrinsic signals bear greater weight on the regulation of hormone secretion from alpha cells compared to beta cells (37); however, these mechanisms are much less well characterized and not yet fully understood. It has been suggested that, in contrast to beta cells, the depolarization of the plasma membrane in alpha cells leads to the inactivation of ion channels (38,39). Another signal with seemingly opposite effects in the two cell types is mediated by the incretin hormone GLP-1; GLP-1 enhances calcium transients in beta cells, stimulating insulin secretion (40), while it inhibits calcium currents and hence glucagon secretion from alpha cells (30,41).

Although cell-specific mechanisms may underlie hormone secretion from the different hormone-secreting cells, the basic cellular mechanisms through which hormone secretion occurs appear to be similar in at least alpha and beta cells. The exocytotic machinery of incretins is less studied. Secretory granules containing hormone exist in a readily available pool within the cells (42,43). The trigger for the exocytotic machinery is the entry of bivalent calcium ions into the cytosol through voltage dependent channels, which via a plethora of intracellular changes

ultimately leads to docking of hormone-containing granules and secretion of hormone from the cell (Figure 1) (43). Moreover, all these cells sense surrounding glucose levels (44-46).
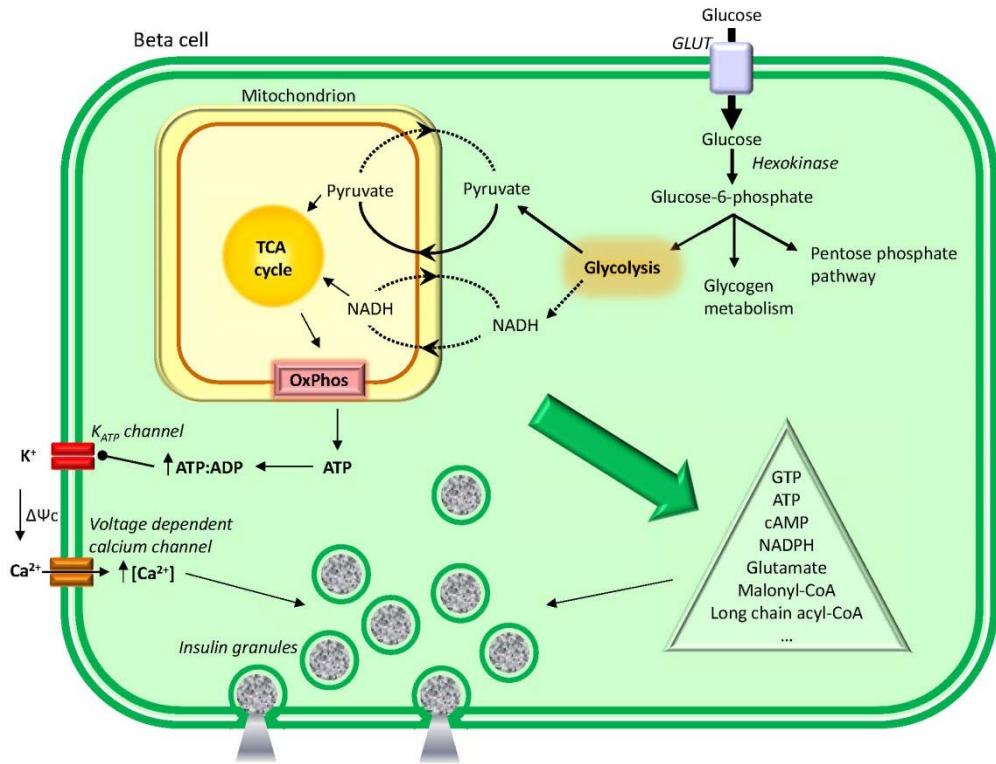
Figure 1: Glucose metabolism and insulin secretion; triggering and amplifying pathways
Glucose metabolism in the pancreatic beta cell ultimately results in insulin release from the cell. There are two active pathways in beta cells, which result in insulin secretion. The first is the triggering pathway, which is the direct trigger of glucose metabolism; closure of ATP-sensitive potassium channels (KATP-channels) and membrane depolarization allowing bivalent calcium ion influx triggering insulin granules to fuse with the plasma membrane. The second pathway potentiates insulin secretion independent of the potassium channel activity. Dashed arrows indicate cycling and shuttle activity.

The role of the incretin GLP-1

GLP-1 is an incretin, or gut hormone, secreted from intestinal L cells in response to the presence of nutrients, such as lipids (47), amino acids (48) and sugars (46), in the lumen. Incretins act on the endocrine system in several ways. They have a preparatory role providing signals, which prime the system to decrease circulating plasma glucose levels after a meal (49). They serve to enhance the response (50) and have a long term role in that they stimulate differentiation (51) and prevent

apoptosis of beta cells (52). Despite a half-life of less than 2 minutes in circulation, GLP-1 is a potent stimulator of insulin secretion from beta cells and suppressor of glucagon secretion (53) from alpha cells. As GLP-1 only potentiates insulin secretion in the presence of elevated blood glucose levels it is incapable of inducing severe hypoglycemia (54,55), a potentially fatal complication of insulin treatment. In addition to directly stimulating insulin secretion in beta cells, peripherally secreted GLP-1 is thought to have the ability to both stimulate nerves locally and indirectly influence beta cells through the nervous system (56). Since GLP-1 has the ability to restore glucose sensitivity (57), promote cell differentiation, increase insulin biosynthesis (58) and prevent apoptosis in beta cells, it may improve the ability of an individual to maintain glucose homeostasis.

L cells have similar secretory control as beta cells; i.e., a biphasic pattern of secretion (59,60) generally associated with both a triggering and amplifying pathway. Importantly, these cells have additional control mechanisms not found in beta cells; in addition to GLUT1/2, L-cells express electrogenic amino acid (48) and sugar transporters (61). Hence, sodium-coupled nutrient uptake has been shown to account for a large proportion of GLP-1 secretion.

Glucose metabolism; production of energy, essential substrates and coupling factors

Glycolysis; versatile energy production

Glycolysis breaks down glucose-6-phosphate to form pyruvate. This can occur under both aerobic and anaerobic conditions, providing a means of energy production when respiration fails. The net result of aerobic glycolysis is the production of reducing equivalents for mitochondrial metabolism, generation of pyruvate and energy. The products of glycolysis provide substrates for further energy production as well as reducing equivalents for a host of mechanisms in the cell.

The central role of mitochondrial metabolism

Mitochondria are organelles in mammalian cells that produce the majority of energy required for normal cellular function. Mitochondria are thought to have originally been a symbiotic species since they are surrounded by a double membrane and contain their own circular DNA molecule. Of pivotal importance to

both energy production and biosynthesis is the amphibolic TCA cycle; occurring in the mitochondrial matrix, it results in the complete oxidation of metabolites, generation of substrates for biosynthesis and reducing equivalents. Moreover, the mitochondria play an important role in hormone secretion as they convert secretagogues into energy and signaling molecules driving and maintaining hormone release (62).

Pyruvate is converted into either acetyl-coenzyme A (acetyl-CoA) by pyruvate dehydrogenase (PDH) or oxaloacetate by pyruvate carboxylase (PC), the latter serving as the carrier of acetyl groups in the TCA-cycle. A passage through the TCA cycle fully oxidizes the acetyl groups producing energy, reducing equivalents, carbon dioxide and water. The reducing equivalents are utilized for further energy production through oxidative phosphorylation; proteins embedded in the inner mitochondrial membrane, known as the electron transport chain (ETC), generate a proton gradient across the membrane and ATP synthase employs the resulting electrochemical gradient in the production of ATP (63).

The TCA cycle also plays a central role in biosynthesis; intermediates can be extracted as substrates for generation of biomolecules such as fatty acids and amino acids in cataplerotic processes. Cataplerosis has the potential to reduce the relative activity of the TCA cycle and as such impact the secretory ability of the cells. As a counter, anaplerosis serves to replenish TCA cycle intermediates enabling continued metabolic flux essential for normal cellular function and hormone secretion (64,65). One of the main anaplerotic steps is the previously mentioned conversion of pyruvate to oxaloacetate by PC. Amino acid metabolism can also provide substrates for anaplerotic processes and as such can be potent activators of hormone secretion (66).

One such amino acid is glutamate. It can be converted to α-ketoglutarate by glutamate dehydrogenase (GDH). GDH activity is regulated both allosterically and covalently by e.g., ADP ribosylation, catalyzed by sirtuin 4 (SIRT4), and allosterically by leucine and GTP (67). A combination of an allosteric GDH activator together with glutamine, a substrate for glutamate synthesis, results in robust insulin secretion (68). In addition to activating GDH, leucine can be metabolized into acetyl-CoA via alpha ketoisocaproic acid, also fueling the TCA cycle.

Pyruvate cycling, generation of signaling molecules

Three cycles involving the transport of pyruvate into the mitochondria have been outlined in beta cells: pyruvate-citrate, pyruvate-isocitrate and pyruvate-malate cycles (16). In addition to transporting pyruvate into the mitochondria, these cycles have been suggested to generate coupling factors for GSIS: NADPH, a

potential coupling factor for GSIS (19), and malonyl-CoA, a mediator in fatty acid potentiated insulin secretion (24,69). The pyruvate-citrate and isocitrate cycles rely on the export of citrate from the TCA-cycle into the cytosol. In the pyruvate-citrate cycle, citrate is converted to oxaloacetate and acetyl-CoA. Oxaloacetate is then converted to pyruvate by malic enzyme in the NADPH-generating step, and pyruvate may re-enter the TCA-cycle. Acetyl-CoA in turn is utilized in lipogenesis. In the pyruvate-isocitrate cycle, citrate is converted to isocitrate, which is exported from the mitochondria and in turn converted to α-ketoglutarate in a NADPH-generating step; α-ketoglutarate then re-enters the mitochondria to fuel the TCA-cycle. Finally, in the pyruvate-malate cycle malate is exported from the mitochondria and converted to pyruvate in an NADPH-producing step by malic enzyme. Pyruvate cycling and key enzymes involved in it have been linked to GSIS in clonal beta cells (70,71) but with less evidence in islets (72).

Mitochondrial shuttles, providing reducing equivalents for oxidative phosphorylation

The partial oxidation of glucose in glycolysis results not only in production of pyruvate for mitochondrial energy production but also in increased cytosolic levels of reducing equivalents. An accumulation of NADH in the cytosol would eventually inhibit glycolysis, and thereby halt downstream triggering reactions for insulin release. There are no mitochondrial transporters of NADH. Instead, to ensure continued glycolytic activity, two mitochondrial shuttle systems have evolved, which indirectly transport reducing equivalents across the inner mitochondrial membrane: the malate-aspartate and glycerol-phosphate shuttles. The malate-aspartate shuttle directly impacts mitochondrial activity as well as insulin secretion (73,74). The glycerol-phosphate shuttle is redundant to the malate-aspartate shuttle in beta cells and the loss of both shuttles is required for inhibition of insulin secretion (23).

In the malate-aspartate shuttle, oxaloacetate is converted into malate in the cytosol, a reaction requiring the oxidation of NADH to yield NAD+. Malate is transported into and α-ketoglutarate is transported out of the mitochondrial matrix by the malate α-ketoglutarate antiporter. In the mitochondrial matrix, malate is oxidized to yield oxaloacetate, while NAD+ is reduced to NADH. NADH is then oxidized in the electron transport chain. The process continues in the mitochondria with the conversion of oxaloacetate into aspartate and the conversion of glutamate into α-ketoglutarate by a mitochondrial version of aspartate aminotransferase (AAT). Aspartate is then exported from the mitochondria and glutamate imported by the glutamate-aspartate antiporter. In the cytosol the reverse reaction occurs, catalyzed by the cytosolic version of AAT, producing the starting materials oxaloacetate and

glutamate (Figure 2). Glutamate, an intermediate of this shuttle system, has been implicated as coupling factors for glucose-stimulated insulin secretion (8). The glycerolphosphate shuttle is principally simpler than the malate-aspartate shuttle. Briefly, the glycolytic intermediate dihydroxy acetonephosphate is reduced to yield glycerol 3-phosphate. Then, at the mitochondrial inner membrane, glycerol 3-phosphate is converted back into dihydroxy acetonephosphate by mitochondrial glycerol 3-phosphate dehydrogenase, producing one $FADH_2$ in the mitochondrial matrix. Hence, one cytosolic NADH is converted to one mitochondrial $FADH_2$. Consequently, there is energy loss in this shuttle.
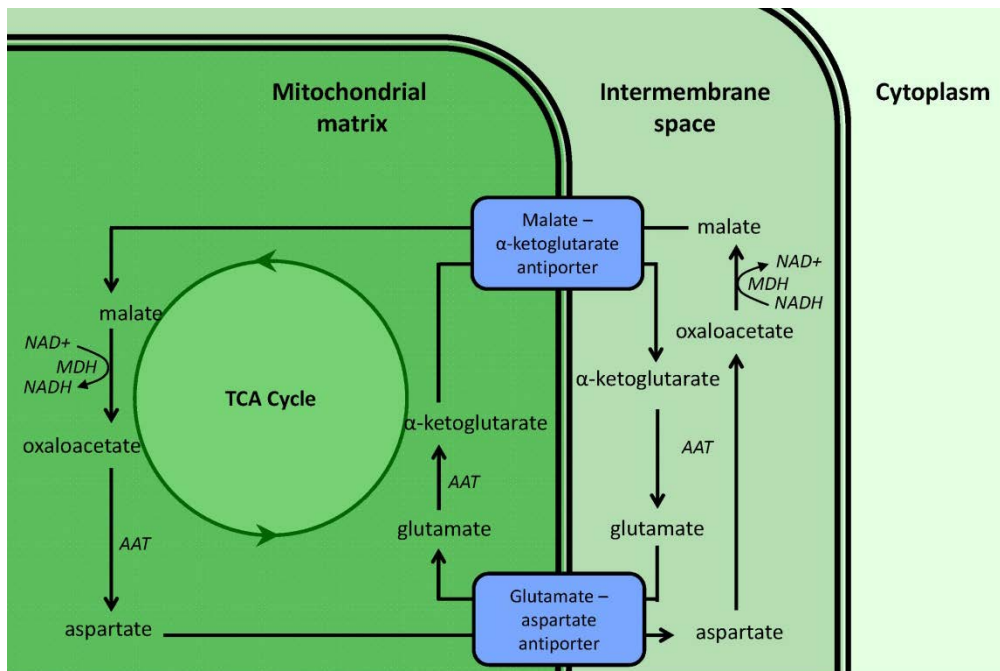
Figure 2: Mitochondrial malate-aspartate shuttle
The inner mitochondrial membrane is impermeable to reducing equivalents (NADH) produced in glycolysis. The malate-aspartate shuttle provides a means to transport the reducing power from the cytoplasm to the mitochondrial matrix.

Implication of non-oxidative glucose metabolism in GSIS

Glucose metabolism is central to insulin secretion in beta cells as described; however, glycolysis is not the only fate of glucose in cells. In addition, two highly conserved metabolic pathways use the same starting substrate of glucose-6-phosphate: the pentose phosphate pathway and glycogenesis.

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The Pentose Phosphate Pathway

The pentose phosphate pathway, also known as the phosphogluconate pathway, produces substrates for the production of nucleotides and aromatic amino acids. It also produces reducing equivalents in the form of NADPH, supplying as much as 60% of the NADPH needed in humans. NADPH is used for anabolic reactions and prevention of oxidative stress. Despite studies implicating NADPH in GSIS (75-77), it was widely thought that the pentose phosphate pathway was inactive in the beta cell (78,79), the source of NADPH being the pyruvate cycles discussed previously. Recently, this pathway has been linked to the regulation of insulin secretion (80-82).

Glycogen metabolism

Glycogenesis is responsible for the majority of glucose-lowering activity of liver and skeletal muscle in response to insulin. It converts glucose-6-phosphate, through a number of enzymatic reactions, to glycogen, a stable, readily available, energy source for muscle and hepatic cells. The enzymes involved in glycogen metabolism are regulated by epinephrine, glucagon, insulin and calcium ions in muscle (83-86). Despite shared glucose sensing enzymes with hepatic cells (GLUT2 and glucokinase), glycogen accumulation in beta cells has been thought to be a pathogenic response to hyperglycemia. Studies have shown glycogen in islets and beta cells of diabetic models and humans with diabetes (87-91). Despite glycogen metabolism potentially interfering with the consensus model of supply driven oxidative metabolism in beta cells, glycogenesis and glycogenolysis have not been widely studied in beta cells or islets.

Diabetes Mellitus – failure of glucose homeostatic control

Glucose is likely the most important substrate in human metabolism; this simple sugar provides the raw material required to produce not only energy but is also the building block of many of the essential substances our bodies rely on for survival. A crisis in this essential system could have dire short and long term effects for an individual's survival. Diabetes mellitus is the condition incurred when the endocrine system cannot control plasma or blood glucose levels, resulting in a rise in circulating glucose levels associated with a reduction in glucose uptake in most tissue. The disease has often been classified into two distinct diseases: type 1 diabetes (T1D), or childhood/autoimmune diabetes and type 2 diabetes (T2D), or age/lifestyle diabetes (92). Although the underlying mechanisms for the inception of the disease are largely different the end result is uncontrolled plasma glucose

levels. T1D, which occurs in childhood, is thought to have a genetic predisposition, but requires an environmental trigger, resulting in the destruction of pancreatic beta cells by an autoimmune response. Although the genetic predisposition to T2D is strong (93) the disease is less acute than T1D and often develops over many years. In patients with T2D, pancreatic beta cells are largely intact but become progressively less responsive to elevated plasma glucose levels in conjunction with increased insulin resistance; peripheral tissues are less responsive to the actions of insulin, lowering the effectiveness by which they take up glucose from the blood. In response to the extended periods of hyperglycemia, the pancreatic beta cells respond by increasing the amount of insulin secreted. As the disease progresses, beta cells eventually fail to secrete sufficient amounts of insulin to maintain glucose homeostasis, due to failed responsiveness to glucose and, to a lesser extent, loss of beta cell mass (94-97). In addition to T1D and T2D, there are several less common types of diabetes, including latent autoimmune diabetes in adults (LADA) (98) and maturity-onset diabetes in the young (MODY) (99). LADA is a slowly developing autoimmune type of diabetes in older individuals. MODY is the result of genetic mutations in specific key genes and is often referred to as monogenic diabetes. The severity and progression of the disease is dependent on the type of MODY, with some possible to treat with life-style intervention alone (100).

Type 2 Diabetes – failure of endocrine signals

T2D is a progressive disease; it is the most commonly occurring and by far the most rapidly growing of all diabetes types, with estimations of close to 600 million affected individuals by the year 2035 (101). It is estimated that almost half of the individuals with T2D are unaware they have the disease and may live several years without diagnosis, which leads to greater risk for future severe complications and poorer long term prognosis. T2D is a complex condition developed due to a combination of life-style choices, environmental and genetic aspects, which result in a perturbation of the influx and removal/uptake of glucose from the blood stream. It is argued whether insulin resistance or defects in insulin secretion is the main factor precipitating the disease (102) however, as most people develop insulin resistance with age, but not all develop T2D, failure of the beta cells to compensate for increased insulin resistance with increased release of insulin is the ultimate trigger of the disease.

The control of glucagon secretion also falters during T2D; hypersecretion of glucagon contributes to hyperglycemia (103) and latter perturbed secretion of the hormone could increase the risk of severe hypoglycemia (28), a severe complication of diabetes. There is growing evidence that dysregulation of glucagon secretion plays an important role in exasperating disease onset and

progression (104). Interestingly, if both insulin and glucagon signaling are blocked normal blood glucose levels are maintained in mice (105).

Earlier studies suggested that also GLP-1 secretion was perturbed in subjects with T2D; postprandial levels of the incretin were shown to be reduced (106-108) and L cells too suffer from insulin resistance, resulting in reduced insulin-stimulated GLP-1 secretion (60); however, recent studies have challenged this view, suggesting that changes in GLP-1 may vary between individuals (109).

Treatment options for diabetes

There is a multitude of treatment options for the disease, depending on type, severity and disease progression. In general, first line of treatment for T2D should include life-style and/or diet intervention. It has been shown that reduced caloric intake (600 kCal/day) can normalize fasting plasma glucose levels, beta cell function and hepatic insulin sensitivity (110). Dietary and life-style intervention therapy are difficult to perpetuate and do not often lead to sustained benefits (111). An alternate means to restrict caloric intake and elicit weight loss is bariatric surgery, which also results in remission of T2D (112). In the case of T1D treatment, insulin substitution is required. Other options include islet transplantation and perhaps in the future, beta cell replacement derived from stem cells.

For most patients pharmaceutical treatment is required at some point during disease progression. Pharmaceuticals target various aspects of the disease; some stimulate insulin secretion (sulfonylureas/meglitinides) or mimic hormone action (GLP-1 analogues), while others reduce or inhibit processes; reducing the amount of glucose produced by the liver or lowering of insulin resistance (biguanides/thiazolidinediones), preventing degradation of GLP-1 (DPP-4 inhibitors), reducing kidney reabsorption of glucose (SGLT2 Inhibitors) or blocking the intestinal breakdown of carbohydrates (alpha-glucosidase inhibitors). Ultimately, the final line of T2D treatment is insulin replacement therapy. One of the most feared complications of diabetes treatment is hypoglycemia, which is potentially fatal. Naturally, insulin injections pose the biggest threat but also drugs that directly stimulate the beta cells to secrete more insulin have the potential to cause hypoglycemia. As such, preferred treatments involve medication that targets secondary pathways to achieve glucose homeostasis, such as GLP-1 augmentation.

Aims

- To evaluate in vitro metabolic models for the study of beta cell function (studies I and II)

- To evaluate similarities and differences in stimulus-secretion coupling of different glucose-sensing endocrine cells (studies III and IV)

- To investigate a response associated with T2D progression; glycogen metabolism in beta cells (study V)

General Methodology

Models of endocrine cell metabolism

Studies of human diseases rely on the use of various types of experimental models in order to efficiently test hypotheses. Models provide platforms of study without requiring primary material or samples. Moreover, they provide more controlled systems and more easily manipulated systems. In order for a model to be of value, it needs to be as similar to the in vivo environment as possible and also respond and function in a similar manner in a controlled laboratory environment. Researchers utilize in vitro and in vivo models as substitutes for biological function. Both model types have their benefits and draw-backs but generally speaking, in vitro models are used in the initial stages as in vivo models tend to be more costly, complex and carry an ethical burden.

In vitro models in endocrine cell research

We utilized a variety of immortalized cell cultures for the investigations: a murine (rat) insulinoma cell line, INS-1 832/13 (113); a human embryonic beta cell line, EndoC-βH1 (114); a murine (mouse) derived alpha cell line, αTC1-6 (115) and a murine (mouse) derived L-cell line, GLUTag (116). The INS-1 832/13 cells were derived from a parental cell line, INS-1, which was obtained from an x-ray induced insulinoma. It has a functional copy of the human insulin gene inserted into its genome. The EndoC-βH1 cells were obtained by transducing human embryonic tissue with a lentivirus expressing simian vacuolating virus 40 large T antigen (SV40LT) under the control of the insulin promoter. This produced cells that express the oncogene concurrently with the insulin promoter allowing the cells to retain proliferative characteristics while producing and secreting insulin glucose-dependently. αTC1-6 cells were derived from a parental cell line, alpha tumor cell-1, established from glucagonoma in mice, but which produces both glucagon and insulin. Clone 6 of this cell line is more differentiated, it does not express insulin and has higher expression of glucagon than the parental line (117). GLUTag cells were isolated from an enteroendocrine cell tumor expressing the

proglucagon gene. GLUTag cells express genes consistent with enteroendocrine differentiation, and secrete GLP-1 in vitro (118).

Islets from mice and rats are often used as models, which may provide a more faithful representation of function as the islets maintain internal signaling and cell to cell interaction. The importance of cell-cell contact has been revealed in studies showing enhanced performance of pseudo-islets as opposed to dispersed islet cells (119,120), perhaps due to synchronization of cells via gap junctions (121,122). Results from investigations in islets are representative of the islet structure as a whole but results may not be attributed to any single cell type. In studies I through III we utilized rat, mouse or human islets as a basis for comparison to in vitro cell cultures, primarily to verify discoveries made in cell lines.

Human islets of Langerhans were used as a 'gold standard' comparison in study I and V. Isolated islets from human donors provide the benefits of intact islet structure and a human genetic background, both of which may be required for detailed and relevant studies. These islets were obtained from the Nordic Center for Clinical Islet Transplantation at Uppsala University, which process material from deceased donors, primarily for clinical use but also for research. The material was processed in house by the Human Tissue Laboratory at our diabetes center before being provided as purified islet extracts.

Gene expression and RNA interference

To evaluate messenger RNA (mRNA) levels the quantitative polymerase chain reaction (qPCR) was used, in conjunction with western blotting to evaluate the effect of RNA interference on target proteins expression. Taqman qPCR utilizes exonuclease activity of Taq polymerase to cleave a labeled probe hybridized to its homologous sequence. Once cleaved the probe loses its quencher resulting in a fluorescence signal that can be quantitatively measured. In western blotting, protein specific antibodies are used to identify proteins that have been separated by gel electrophoresis based on size. The proteins are transferred from the gel to a polyvinylidene fluoride (PVDF) membrane by means of electrophoresis. The membrane is then exposed to antibodies that bind to target proteins and can be visualized using secondary antibodies and detection reagents.

RNA interference, employing short interfering RNA (siRNA), is a commonly used method to reduce gene expression of target genes without the need of genetic mutation. In this method, a short double stranded RNA sequence is carried by a multiprotein RNA-induced silencing complex (RISC) as a template to recognize complementary mRNA transcripts, which it cleaves (123). This causes mRNA

degradation and prevents protein translation, hence significantly lowering the presence of the protein within the cell. To introduce the siRNA into the cells, we employed lipid-mediated transfection, in which the siRNA is 'packaged' into cationic lipid structures, liposomes, in complex with the anionic RNA. The liposomes fuse with the plasma membrane providing the siRNA entry into the cytoplasm through endocytosis.

Functional and Metabolic Analysis

In order to evaluate the usefulness of an in vitro model, we examined whether cell-specific phenotypes and function were retained in culture. The assays performed focused on function (hormone secretion) and the underlying mechanism (metabolism).

Cellular respiration

The oxygen consumption rate (OCR) offers a good estimate of oxygen-dependent energy production in cells. In the case of beta cells, this represents an essential metabolic function as oxygen consumption by the ETC is a prerequisite for mitochondrial ATP production. To measure changes in the oxygen tension resulting from cellular metabolism over time, we utilized the Seahorse XF24 Extracellular Flux Analyzer. By adding a number of chemical compounds, it can be used to identify effects on specific parts of the respiratory chain (63). Post initial stimulatory addition, oligomycin is generally added to the system, blocking ATP synthase and halting ATP production through oxidative phosphorylation, enabling the measurement of the inherent proton leak and the oligomycin-sensitive ATP turnover. Next, carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) is added, causing uninhibited proton leak over the inner mitochondrial membrane, allowing estimation of maximal respiratory rate. Finally, when rotenone is added, the electron transport chain is interrupted through the prevention of electron transfer between complex I and ubiquinone, halting mitochondrial ATP production and resulting in a measure of non-mitochondrial respiration.

Metabolite profiling

Gas chromatography (GC) joint with mass spectrometry (MS) provides a powerful tool to measure levels of intracellular metabolites (124,125). In the GC,

metabolites are separated based on their volatility. The eluting analytes are then detected by MS, allowing for quantification and identification of the compounds based on their mass and fragmentation pattern. As these processes require analytes to be in the gas phase, they must remain stable when vaporized, limiting compounds that can be analyzed. Samples are derivatized, to alter functional groups on the analytes, to lower the boiling point and to improve the thermal stability to achieve this in a greater number of compounds. Despite these limitations, the GC/MS method in our hands normally allows analysis of approximately 70 metabolites, including the majority of metabolites in central glucose metabolism.

Analysis of metabolites extracted from cellular lysates after different treatments yields a snapshot of the state of cellular metabolic processes. The method does not discriminate between metabolic flux and accumulation so differences may be the result of either. This notwithstanding, metabolite profiling may still be a powerful tool for generation of hypotheses (80,126,127).

Statistical methods

Changes in metabolism elicited by a single fuel may be very complex, involving changes in numerous metabolites and pathways. Hence, a traditional analysis and representation of the data may be insufficient. Multivariate statistics gives us tools that facilitate analysis of large data sets that contain covariation, has the ability to visualize complex patterns and to detect outliers, all leading to improved understanding of the information contained in such data (128). In our studies, we applied orthogonal projections to latent structures discriminant analysis (OPLS-DA) (129) to analyze data and then visualized the data, using both score scatter and shared-and unique structures (SUS)-like plots (130) with significant differences derived from jack-knifed confidence intervals of the loading vectors (131). In a score scatter plot, each point represents the variation of all variables measured in a sample and allows us to identify experimentally driven cluster patterns. In SUS-like plots, the correlations from the OPLS-DA models are plotted allowing visualization of differences shared between or unique to any of the two conditions, as compared to a reference condition (Figure 3).

Figure 3: How to interpret SUS-like plots
In the SUS-plot, two experimental conditions (e.g., two stimulatory conditions) are individually compared to a reference conditions (e.g., basal glucose levels), using, e.g., OPLS-DA. The correlations obtained from the resulting models are plotted against each other for a number of experimentally determined variables (e.g., metabolites). The plot is divided into nine areas based on the significance of these correlations (i.e. if the change in levels of a metabolite was significantly different between two treatments, such as low and high glucose stimulation). The X- and Y-axes are hence divided into three segments: increase in levels (right or up), no change (middle) and decreased levels (left or down). As such the center panel of the plot encompasses variables that were not significantly changed by the treatment and the outer panels showing either shared (an increase or decrease in both conditions) or unique (only increased or decreased in one condition) attributes.

Results and Discussion

Evaluation of in vitro models for investigations on beta cell metabolism (Study I and II)

In the first study, we had the fortune of acquiring early access to a much needed tool in the field: a stable human beta cell line, EndoC-βH1 (114). The first step in evaluating new models is to characterize their phenotypic function and compare them to current 'gold standards' in the field. The aim was to establish basic functional similarities and differences to current beta cell models and evaluate the utility of this newly established cell line. Hence, we compared the EndoC-βH1 cells to an accepted and well-established murine β-cell model, INS-1 832/13 cells, and finally validated key findings in human islets.

We studied the functional and metabolic effect of glucose stimulation on the cells, and found that such stimulation elicited similar changes in insulin secretion, glucose utilization, respiratory rates and metabolite profiles in both cell lines (Figure 4), although overall the magnitude of response was lower in EndoC-βH1. The most noticeable difference was that glucose failed to provoke the characteristic oscillatory fluctuations in the free cytoplasmic calcium levels and plasma membrane potential seen in INS-1 832/13 and primary beta cells (Figure 5). Despite the absence of these characteristics, depolarization of the plasma membrane with KCl provoked robust insulin secretion, indicating that the exocytotic machinery is intact in the EndoC-βH1 cells (Figure 5).

One possible explanation for the lack of oscillations in the EndoC-βH1 may lay in their fetal nature. Beta cells are not considered to be true endocrine cells until the end of the first trimester of human pregnancy (132). As such fully developed electrophysiology may not be required of early fetal beta cells. Expression levels of certain voltage-dependent calcium channels in mature beta cells differ to that of fetal beta and alpha cells (133).

A perpetual problem with in vitro models is the manipulation of the primary tissue required to produce the model. In the case of cell lines, immortalization is required for sustained proliferation in culture, permanently changing the phenotype of the cells to a more cancerous one. In the case of isolated islets, the time for the
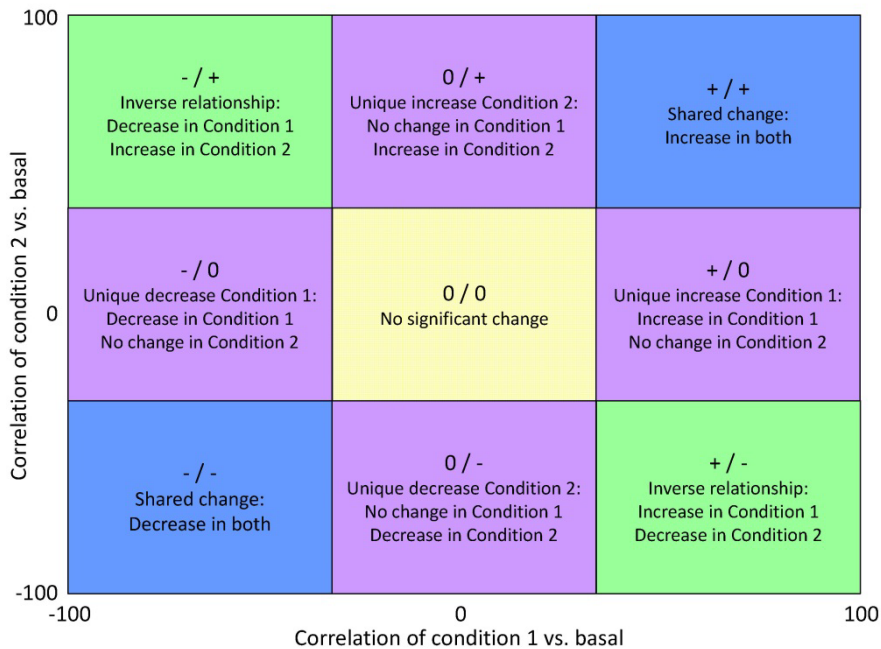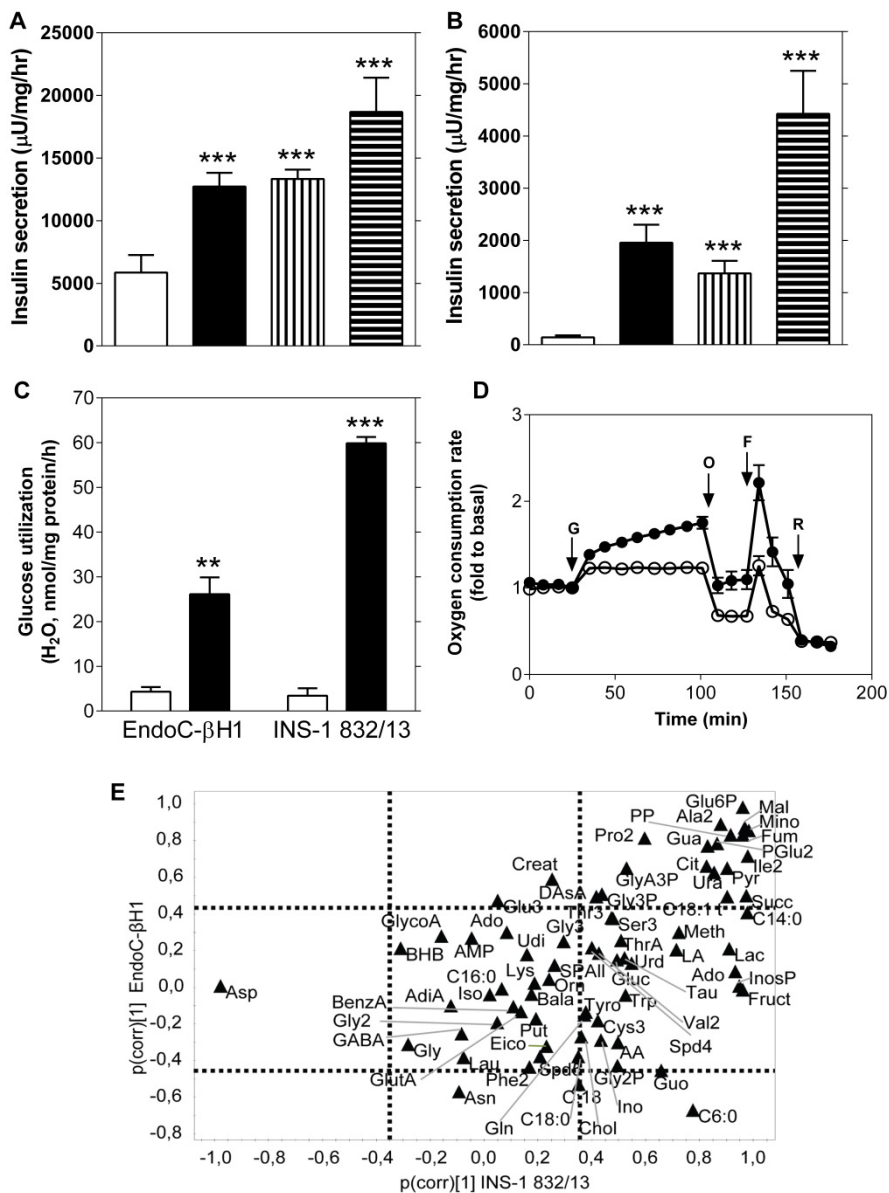
Figure 4: How to interpret SUS-like plots

In the SUS-plot, two experimental conditions (e.g., two stimulatory conditions) are individually compared to a reference conditions (e.g., basal glucose levels), using, e.g., OPLS-DA. The correlations obtained from the resulting models are plotted against each other for a number of experimentally determined variables (e.g., metabolites). The plot is divided into nine areas based on the significance of these correlations (i.e. if the change in levels of a metabolite was significantly different between two treatments, such as low and high glucose stimulation). The X- and Y-axes are hence divided into three segments: increase in levels (right or up), no change (middle) and decreased levels (left or down). As such the center panel of the plot encompasses variables that were not significantly changed by the treatment and the outer panels showing either shared (an increase or decrease in both conditions) or unique (only increased or decreased in one condition) attributes.

Figure 5: Free cytoplasmic calcium levels and plasma membrane potential in EndoC-βH1 and INS-1 832/13 cells
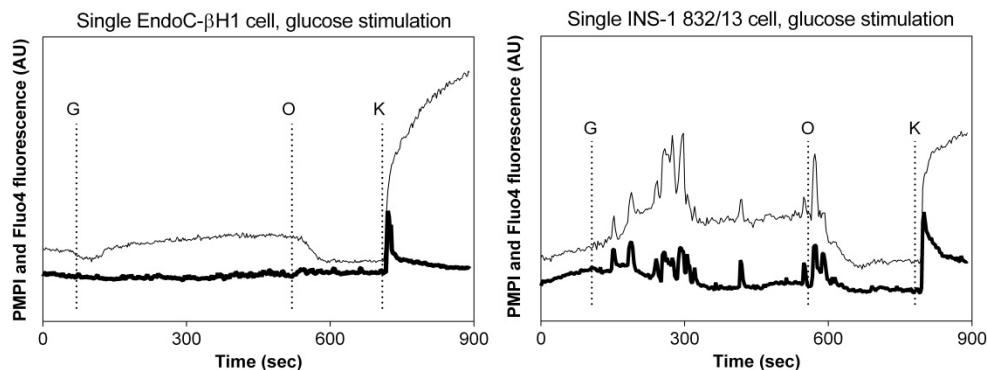Single cell measurements of the free cytoplasmic calcium (bold line) and plasma membrane potential (thin line) in EndoC-βH1 and INS-1 832/13 cells. The additions made were 16.7 mM glucose (G) 0.5 ng/µL oligomycin (O) and 25 mM KCl (K).

processing of the material and the lack of ability to provide essential nutrients without a circulatory system result in islet stress, altered gene expression (134) and onset of necrosis (135). Despite these drawbacks, both models are extensively used in studies to determine biological function and disease progression. In study II, we set out to compare the extensively utilized INS-1 832/13 rat beta cell model (113) with isolated primary rat islets to evaluate potential fundamental differences, which may result in deviating results depending on the model used. Gene enrichment analysis of gene expression data showed distinct differences between the two models. In the clonal cells, the enriched pathways can be explained by the aforementioned treatment for generation of the models, namely cell cycle, cancer signaling, biosynthesis and nucleotide replication.

Bearing in mind that a variety of different pathways operate differently in the two models, it is reassuring to see that our data showed similar glucose responses in the two systems, with the main differences being amplitude of responses (Figure 6).

Importance of mitochondrial shuttles for hormone secretion (study III)

For the third study we employed cell lines, sorted islet cells and isolated islets when studying stimulus-secretion coupling in pancreatic alpha cells. Contrary to what may be expected, we found that alpha cells concentration-dependently
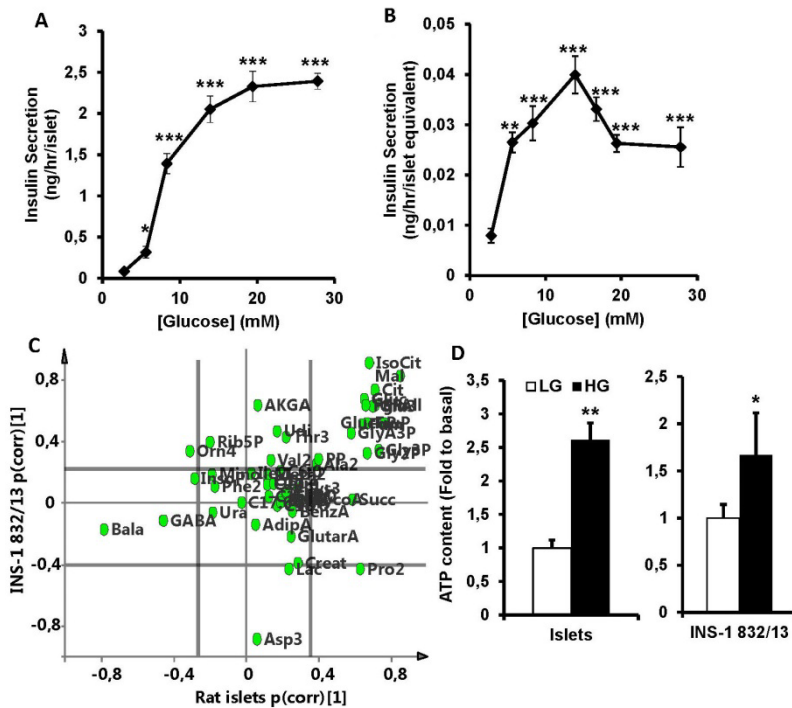
Figure 6: Glucose-elicited metabolic changes in rat islets and INS-1 832/13 cells
Glucose-stimulated insulin secretion (A & B), changes in metabolite profiles (C) and ATP content (D) were all similar in rat islets and INS-1 832/13 cells.

secreted glucagon in response to increasing glucose stimuli. This phenomenon has been observed previously in isolated primary alpha cells as well, and it is believed that the intra-islet communication and paracrine regulation are pivotal to proper alpha cell function and glucagon secretion in vivo (136-138). Such data additionally support the notion that the secretion machinery is similarly designed in both alpha and beta cells. In line with this, closer analysis of metabolism revealed that glycolytic metabolism was similar in alpha and beta cells in response to glucose. Interestingly, metabolic activity downstream of glycolysis appeared less glucose-responsive in alpha cells. As we further investigated this difference, our data confirmed that the mitochondrial shuttles were redundant in beta cells (23), while the glycerol phosphate shuttle appeared essentially inactive in alpha cells; inhibition of the malate-aspartate shuttle in alpha cells and islets abolished glucagon secretion, while insulin secretion from beta cells and islets was sustained (Figure 7). This speaks to the critical role of mitochondrial metabolism in direct response to glucose concentration in beta cells, while regulation of glucagon secretion is under more subtle control.
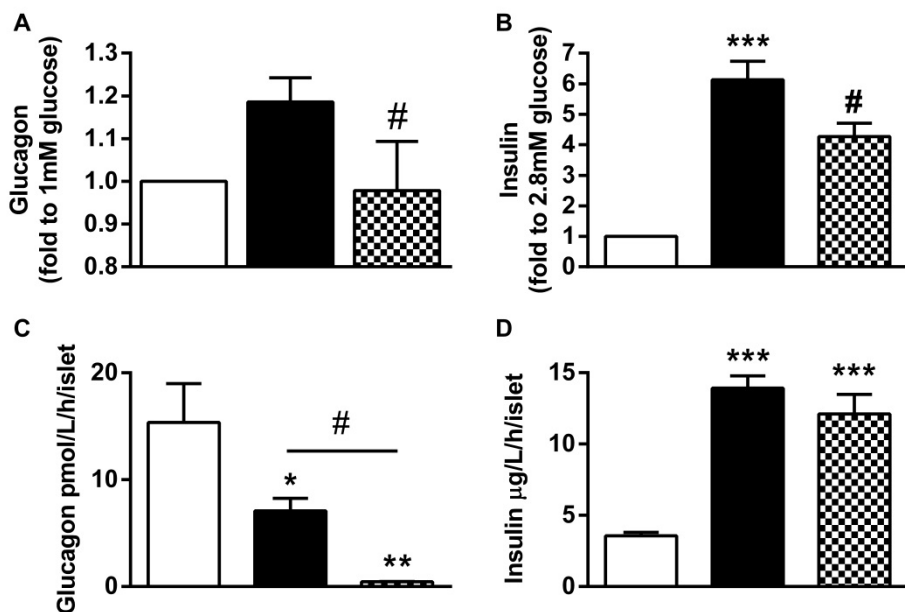
Figure 7: Inhibition of the malate-aspartate shuttle abolishes glucagon secretion but has little impact on insulin secretion
Hormone secretion (glucagon and insulin) was evaluated in clonal cells (A, B) and primary islets (C, D) at basal levels (white bars) and with glucose stimulation in the absence (black bars) or presence of an inhibitor of the malate aspartate shuttle (phenyl succinate, checkered bars).

Mitochondrial metabolism's role in nutrient sensing (study IV)

With the notion that mitochondrial metabolism plays a central role in both beta and alpha cell hormone secretion, we studied the metabolic coupling of GLP-1 secretion from L cells in study IV. Utilizing an established colonic L cell line (GLUTag), we found that mitochondrial metabolism is important to nutrient sensing in L cells. Glucose stimulation provoked increased mitochondrial metabolism and hormone secretion from both cell types. Previous studies have indicated electrogenic transporters playing an important role in L cell stimulus-secretion coupling. Interestingly, we found that stimulation both upstream and downstream of such transporters resulted in qualitatively similar responses in metabolism and hormone secretion. Our data do not rule out such electrogenic nutrient uptake, but indicate that electrogenic nutrient sensing may be of greater importance in the upper small intestine, while alternate regulation may govern GLP-1 secretion in the lower intestine (139).

L cells appear to have differentially regulated amino acid metabolism: a neuron-like glutamate metabolism with a high glutamate dehydrogenase activity, potentially due to intrinsically higher leucine levels. Glutamine, via conversion into glutamate and α-ketoglutarate feeding into the TCA-cycle, elicited increased mitochondrial activity in L cells resulting in robust GLP-1 secretion (Figure 8). In contrast, glutamine stimulation had no effect on insulin secretion from INS-1 832/13 cells in the absence of leucine.
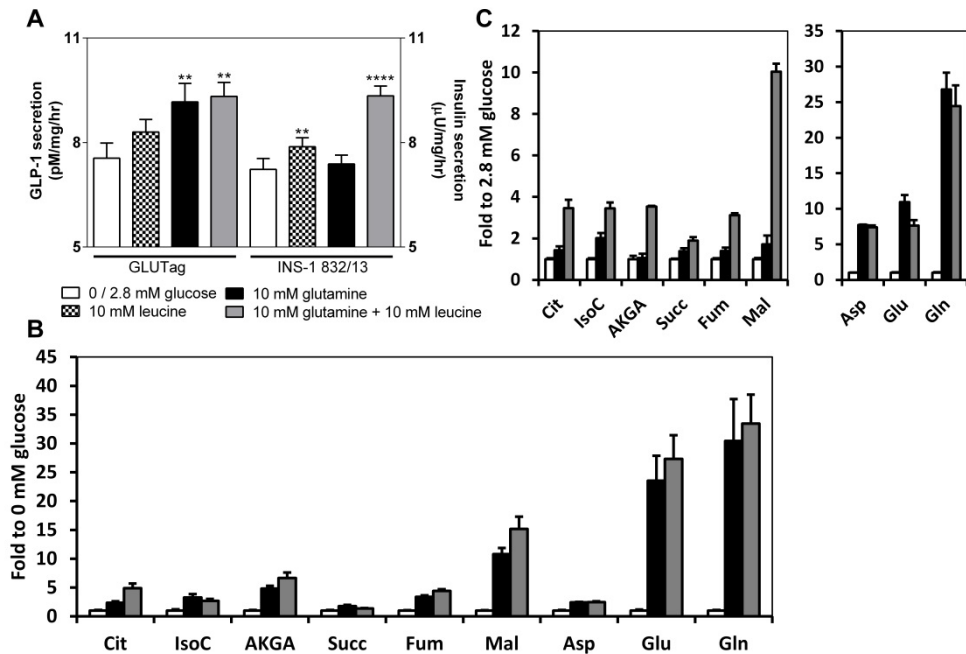
Figure 8: Glutamine activates mitochondrial metabolism and elicits hormone secretion from L cells but not from beta cells
Glutamine (black bars) elicited an increase in hormone secretion from L but not beta cells compared to basal (white bars) (A). The co-stimulation with leucine and glutamine (grey bars) yielded no additional increase in GLP-1 secretion, but significantly increased insulin secretion from beta cells. The same pattern holds true for TCA cycle intermediates in L-cells (B) and beta-cells (C).

Glycogen metabolism in beta cells (study V)

Although highly conserved, glycogen metabolism in beta cells has been considered inappropriate considering the role and function of beta cells in glucose homeostasis; storage of glycogen would provide an internal source for potentiation of insulin secretion during low plasma glucose levels. Such a function would potentially be deleterious to survival. Hence, accumulation of glycogen in the

beta-cell has been considered as a pathological sign possibly associated with diabetes. In our final study, we used beta cell lines and human islets to investigate glycogen metabolism in beta cells. We found that all of the enzymes essential for glycogen metabolism were expressed at the mRNA level in both INS-1 832/13 cells and in human islets. Glycogen levels were associated with glucose concentration (Figure 9) in clonal beta cells and was present in human islets from non-diabetic donors after a 48 hour incubation in medium containing 5.6 mM or 16.7 mM glucose. Together, this illustrates the possibility that glycogen metabolism is active under non-diabetic conditions. In addition, when the enzymes responsible for the production or breakdown of glycogen were inhibited GSIS was reduced. The mechanism by which this occurs is not yet understood, requiring further experimentation.
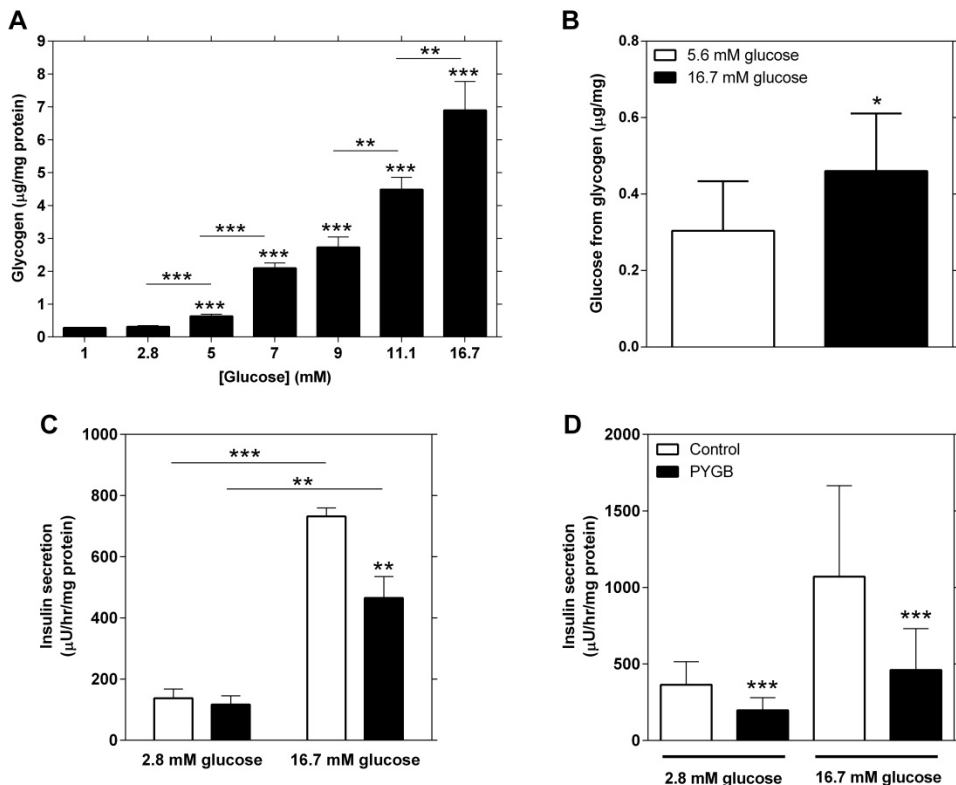

Figure 9: Glycogen metabolism in beta cells
Glycogen accumulated concentration-dependently with increasing glucose concentration in clonal beta cells (A), and was found in human islets after a 48 hour incubation at 5.6 mM or 16.7 mM glucose (B). Silencing of either GYS1 (C) or PYGB (D) reduced glucose elicited insulin secretion.

Summary and major conclusions

Biological systems are complex, as well as redundant, providing researchers with a challenging task when dissecting cause and effect. Moreover, manipulations required for successful experimental design may alter the very nature of the experimental models employed in the search for basic mechanistic understanding, hereby adding to the difficulty. Additionally, "tunnel vision" caused by use of specific cell types, pathways or even proteins of interest may cloud the overall picture of the biological impact a manipulation bears. As such, evaluation and critical analysis of the model systems, along with use of biologically relevant models are critical for generation of reliable and reproducible data. Here, we have evaluated a number of different biological model systems for functional and metabolic similarities and differences. Conclusions from the studies presented are:

- The recently developed human beta cell line EndoC-βH1 provides a research model with qualitatively similar biological function to the previously utilized in vitro models with the added benefit of a human genetic background

- The clonal beta cell line, INS-1 832/13, although cancerous in certain aspects retains similar biological function to that of intact islets

- The malate-aspartate shuttle was identified as pivotal for glucagon secretion from pancreatic alpha cells

- Amino acid metabolism in addition to glucose metabolism drives GLP-1 secretion from L cells

- Glycogen metabolism impacts GSIS in clonal beta cells and reflects ambient glucose concentrations in human islets

Populärvetenskaplig sammanfattning

Sockersjuka, eller diabetes, är en global och växande folksjukdom. År 2014 fanns uppskattningsvis 235 miljoner människor, som var drabbade av sjukdomen, och 2035 tros 440 miljoner vara drabbade. Det finns flera olika typer av diabetes, av vilka typ 2 diabetes (T2D) är den snabbast växande formen. Orsaken till sjukdomen är komplex, men fysisk inaktivitet och övervikt, samt genetiska faktorer är förknippade med T2D. Sjudomen i ett tidigt stadie har få symptom och därmed är det många individer som omedvetet lider av sjukdomen, vilket gör att många inte behandlas förrän allvarliga komplikationer uppstått.

Bukspottskörteln kontrollerar blodsockernivåer. Insulin är ett hormon som frigörs av betaceller i bukspottkörteln och ansvarar för att sänka blodsockernivån efter en måltid. Insulin påverkar muskler och fettceller, vilka i sin tur ökar sockerupptaget och sänker blodsockernivån. Glukagon är ett annan hormon som frigörs av alphaceller i bukspottkörteln. Detta hormon ansvarar för att höja blodsockernivån mellan måltiderna. Glukagon signallerar till leverceller att påbörjar nedbrytning av glykogen till socker, som sedan utsöndras i blodomloppet. Både alpha och betaceller finns i små strukturer i bukspottskörtlen som kallas Langerhanska öar. Om insulin inte frisätts i tillräckliga mängder eller om det förlorar sin effektivitet, så sänks inte blodsockernivån tillräckligt. Om glukagon utsöndras i alltför stora mängder eller om det frisätts när blodsockernivån redan är hög, så tillför det mer socker och höjer blodsockernivån ytterligare. Vid diabetes tros båda dessa hormoner misslyckas med sin uppgift att upprätthålla normala blodsockernivåer.

Biologiska system är extremt komplexa och ett stort antal processer styr även enkla funktioner. På grund av detta är det ofta svårt att studera hur en mekanism fungerar utan att påverka hela systemet, vilket i många fall inte är görbart i människa. För att övervinna detta används modellsystem, såsom cellinjer eller djur. Resultat måste återspegla biologisk funktion, och det är därmed viktigt att modellerna är väl karaktäriserade. I de två första studierna utvärderade vi biologiska modellsystem i form av betacellinjer och öar. Vi visar att funktion och kontroll av ämnesomsättningen i dessa system är jämförbara vilket innebär att de är bra modellsystem att använda för dessa typer av studier.

I den tredje studien visar vi hur väletablerade modeller kan användas för att hitta skillnader och likheter i biologisk funktion. Information om skilda

kontrollmekanismer kan användas för att hitta nya läkemedelsmål för behandling. Vi visar att en specifik cellulär funktion har avsevärt större inflytande på glukagonfrisättning än insulinutsöndring. När vi stoppar denna process i alpha och betaceller hämmas glukagonfrisättning medan insulinsekretion är opåverkad.

I den sista studien jämför vi ämnesomsättning och hormonfrisättning från betacellen med en tarmcellinje som utsöndrar glukagonlik peptid 1 (GLP-1). GLP-1 är en substans som påverkar både insulin- och glukagonfrisättning, men i motsatt riktning. GLP-1 frisätts i närvaro av näringsämnen i tarmen och hjälper då till att öka insulinfrisättning och minska glukagonsekretion för att kontrollera blodsockernivåer. Vi identifierar en unik funktion i tarmcellinjen som möjliggör för dessa celler att känna av även aminosyranivåer, en funktion som saknas i betacellen

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References

1.   Steiner, D. J., Kim, A., Miller, K., and Hara, M. (2010) Pancreatic islet plasticity: interspecies comparison of islet architecture and composition. *Islets* **2**, 135-145

2.   Orci, L., and Unger, R. H. (1975) Functional subdivision of islets of Langerhans and possible role of D cells. *Lancet* **2**, 1243-1244

3.   Elayat, A. A., el-Naggar, M. M., and Tahir, M. (1995) An immunocytochemical and morphometric study of the rat pancreatic islets. *J Anat* **186 (Pt 3)**, 629-637

4.   Wierup, N., Svensson, H., Mulder, H., and Sundler, F. (2002) The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. *Regul Pept* **107**, 63-69

5.   Metz, R. (1960) The Effect of Blood Glucose Concentration on Insulin Output. *Diabetes* **9**, 89-93

6.   Gembal, M., Gilon, P., and Henquin, J. C. (1992) Evidence that glucose can control insulin release independently from its action on ATP-sensitive K+ channels in mouse B cells. *J Clin Invest* **89**, 1288-1295

7.   Sato, Y., Aizawa, T., Komatsu, M., Okada, N., and Yamada, T. (1992) Dual functional role of membrane depolarization/Ca2+ influx in rat pancreatic B-cell. *Diabetes* **41**, 438-443

8.   Maechler, P., and Wollheim, C. B. (1999) Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* **402**, 685-689

9.   Campbell, J. E., and Drucker, D. J. (2013) Pharmacology, physiology, and mechanisms of incretin hormone action. *Cell Metab* **17**, 819-837

10.   Devos, A., Heimberg, H., Quartier, E., Huypens, P., Bouwens, L., Pipeleers, D., and Schuit, F. (1995) Human and Rat Beta-Cells Differ in Glucose-Transporter but Not in Glucokinase Gene-Expression. *Journal of Clinical Investigation* **96**, 2489-2495

11.   McCulloch, L. J., van de Bunt, M., Braun, M., Frayn, K. N., Clark, A., and Gloyn, A. L. (2011) GLUT2 (SLC2A2) is not the principal glucose transporter in human pancreatic beta cells: Implications for understanding genetic association signals at this locus. *Mol Genet Metab* **104**, 648-653

12.   Sansbury, F. H., Flanagan, S. E., Houghton, J. A., Shuixian Shen, F. L., Al-Senani, A. M., Habeb, A. M., Abdullah, M., Kariminejad, A., Ellard, S., and Hattersley, A. T. (2012) SLC2A2 mutations can cause neonatal

diabetes, suggesting GLUT2 may have a role in human insulin secretion. *Diabetologia* **55**, 2381-2385

13. Maechler, P., and Wollheim, C. B. (2001) Mitochondrial function in normal and diabetic beta-cells. *Nature* **414**, 807-812

14. Henquin, J. C. (2009) Regulation of insulin secretion: a matter of phase control and amplitude modulation. *Diabetologia* **52**, 739-751

15. Straub, S. G., and Sharp, G. W. (2002) Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes/metabolism research and reviews* **18**, 451-463

16. Jensen, M. V., Joseph, J. W., Ronnebaum, S. M., Burgess, S. C., Sherry, A. D., and Newgard, C. B. (2008) Metabolic cycling in control of glucose-stimulated insulin secretion. *American journal of physiology. Endocrinology and metabolism* **295**, E1287-1297

17. Wiederkehr, A., and Wollheim, C. B. (2012) Mitochondrial signals drive insulin secretion in the pancreatic beta-cell. *Mol Cell Endocrinol* **353**, 128-137

18. Henquin, J. C. (2000) Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* **49**, 1751-1760

19. Maechler, P. (2002) Mitochondria as the conductor of metabolic signals for insulin exocytosis in pancreatic beta-cells. *Cell Mol Life Sci* **59**, 1803-1818

20. Gembal, M., Detimary, P., Gilon, P., Gao, Z. Y., and Henquin, J. C. (1993) Mechanisms by Which Glucose Can Control Insulin Release Independently from Its Action on Adenosine Triphosphate-Sensitive K+ Channels in Mouse B-Cells. *Journal of Clinical Investigation* **91**, 871-880

21. Detimary, P., Van den Berghe, G., and Henquin, J. C. (1996) Concentration dependence and time course of the effects of glucose on adenine and guanine nucleotides in mouse pancreatic islets. *J Biol Chem* **271**, 20559-20565

22. Watkins, D. T., and Moore, M. (1977) Uptake of NADPH by islet secretion granule membranes. *Endocrinology* **100**, 1461-1467

23. Eto, K., Tsubamoto, Y., Terauchi, Y., Sugiyama, T., Kishimoto, T., Takahashi, N., Yamauchi, N., Kubota, N., Murayama, S., Aizawa, T., Akanuma, Y., Aizawa, S., Kasai, H., Yazaki, Y., and Kadowaki, T. (1999) Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion. *Science* **283**, 981-985

24. Prentki, M., Vischer, S., Glennon, M. C., Regazzi, R., Deeney, J. T., and Corkey, B. E. (1992) Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. *J Biol Chem* **267**, 5802-5810

25. Henquin, J. C., Ravier, M. A., Nenquin, M., Jonas, J. C., and Gilon, P. (2003) Hierarchy of the beta-cell signals controlling insulin secretion. *European journal of clinical investigation* **33**, 742-750

26. Dunning, B. E., and Gerich, J. E. (2007) The role of alpha-cell dysregulation in fasting and postprandial hyperglycemia in type 2 diabetes and therapeutic implications. *Endocrine reviews* **28**, 253-283

27. Quesada, I., Tuduri, E., Ripoll, C., and Nadal, A. (2008) Physiology of the pancreatic alpha-cell and glucagon secretion: role in glucose homeostasis and diabetes. *The Journal of endocrinology* **199**, 5-19

28. Cryer, P. E. (2002) Hypoglycaemia: the limiting factor in the glycaemic management of Type I and Type II diabetes. *Diabetologia* **45**, 937-948

29. Zhou, H., Tran, P. O., Yang, S., Zhang, T., LeRoy, E., Oseid, E., and Robertson, R. P. (2004) Regulation of alpha-cell function by the beta-cell during hypoglycemia in Wistar rats: the "switch-off" hypothesis. *Diabetes* **53**, 1482-1487

30. De Marinis, Y. Z., Salehi, A., Ward, C. E., Zhang, Q., Abdulkader, F., Bengtsson, M., Braha, O., Braun, M., Ramracheya, R., Amisten, S., Habib, A. M., Moritoh, Y., Zhang, E., Reimann, F., Rosengren, A. H., Shibasaki, T., Gribble, F., Renstrom, E., Seino, S., Eliasson, L., and Rorsman, P. (2010) GLP-1 inhibits and adrenaline stimulates glucagon release by differential modulation of N- and L-type Ca2+ channel-dependent exocytosis. *Cell Metab* **11**, 543-553

31. Dupre, J., Ross, S. A., Watson, D., and Brown, J. C. (1973) Stimulation of insulin secretion by gastric inhibitory polypeptide in man. *The Journal of clinical endocrinology and metabolism* **37**, 826-828

32. Adrian, T. E., Bloom, S. R., Hermansen, K., and Iversen, J. (1978) Pancreatic polypeptide, glucagon and insulin secretion from the isolated perfused canine pancreas. *Diabetologia* **14**, 413-417

33. Schmidt, W. E., Siegel, E. G., and Creutzfeldt, W. (1985) Glucagon-Like Peptide-1 but Not Glucagon-Like Peptide-2 Stimulates Insulin Release from Isolated Rat Pancreatic-Islets. *Diabetologia* **28**, 704-707

34. Kreymann, B., Williams, G., Ghatei, M. A., and Bloom, S. R. (1987) Glucagon-like peptide-1 7-36: a physiological incretin in man. *Lancet* **2**, 1300-1304

35. Rorsman, P., and Hellman, B. (1988) Voltage-activated currents in guinea pig pancreatic alpha 2 cells. Evidence for Ca2+-dependent action potentials. *The Journal of general physiology* **91**, 223-242

36. Barg, S., Galvanovskis, J., Gopel, S. O., Rorsman, P., and Eliasson, L. (2000) Tight coupling between electrical activity and exocytosis in mouse glucagon-secreting alpha-cells. *Diabetes* **49**, 1500-1510

37. Gromada, J., Franklin, I., and Wollheim, C. B. (2007) Alpha-cells of the endocrine pancreas: 35 years of research but the enigma remains. *Endocrine reviews* **28**, 84-116

38. Ramracheya, R., Ward, C., Shigeto, M., Walker, J. N., Amisten, S., Zhang, Q., Johnson, P. R., Rorsman, P., and Braun, M. (2010) Membrane potential-dependent inactivation of voltage-gated ion channels in alpha-

cells inhibits glucagon secretion from human islets. *Diabetes* **59**, 2198-2208

39. Spigelman, A. F., Dai, X., and MacDonald, P. E. (2010) Voltage-dependent K(+) channels are positive regulators of alpha cell action potential generation and glucagon secretion in mice and humans. *Diabetologia* **53**, 1917-1926

40. Salapatek, A. M., MacDonald, P. E., Gaisano, H. Y., and Wheeler, M. B. (1999) Mutations to the third cytoplasmic domain of the glucagon-like peptide 1 (GLP-1) receptor can functionally uncouple GLP-1-stimulated insulin secretion in HIT-T15 cells. *Mol Endocrinol* **13**, 1305-1317

41. MacDonald, P. E., El-Kholy, W., Riedel, M. J., Salapatek, A. M., Light, P. E., and Wheeler, M. B. (2002) The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. *Diabetes* **51 Suppl 3**, S434-442

42. Proks, P., Eliasson, L., Ammala, C., Rorsman, P., and Ashcroft, F. M. (1996) Ca(2+)- and GTP-dependent exocytosis in mouse pancreatic beta-cells involves both common and distinct steps. *The Journal of physiology* **496 (Pt 1)**, 255-264

43. Barg, S. (2003) Mechanisms of exocytosis in insulin-secreting B-cells and glucagon-secreting A-cells. *Pharmacology & toxicology* **92**, 3-13

44. German, M. S. (1993) Glucose sensing in pancreatic islet beta cells: the key role of glucokinase and the glycolytic intermediates. *Proc Natl Acad Sci U S A* **90**, 1781-1785

45. Heimberg, H., De Vos, A., Moens, K., Quartier, E., Bouwens, L., Pipeleers, D., Van Schaftingen, E., Madsen, O., and Schuit, F. (1996) The glucose sensor protein glucokinase is expressed in glucagon-producing alpha-cells. *Proc Natl Acad Sci U S A* **93**, 7036-7041

46. Reimann, F., Habib, A. M., Tolhurst, G., Parker, H. E., Rogers, G. J., and Gribble, F. M. (2008) Glucose sensing in L cells: a primary cell study. *Cell Metab* **8**, 532-539

47. Chu, Z. L., Carroll, C., Alfonso, J., Gutierrez, V., He, H. M., Lucman, A., Pedraza, M., Mondala, H., Gao, H., Bagnol, D., Chen, R., Jones, R. M., Behan, D. P., and Leonard, J. (2008) A role for intestinal endocrine cell-expressed g protein-coupled receptor 119 in glycemic control by enhancing glucagon-like peptide-1 and glucose-dependent insulinotropic peptide release. *Endocrinology* **149**, 2038-2047

48. Reimann, F., Williams, L., da Silva Xavier, G., Rutter, G. A., and Gribble, F. M. (2004) Glutamine potently stimulates glucagon-like peptide-1 secretion from GLUTag cells. *Diabetologia* **47**, 1592-1601

49. Knauf, C., Cani, P. D., Kim, D. H., Iglesias, M. A., Chabo, C., Waget, A., Colom, A., Rastrelli, S., Delzenne, N. M., Drucker, D. J., Seeley, R. J., and Burcelin, R. (2008) Role of central nervous system glucagon-like Peptide-1 receptors in enteric glucose sensing. *Diabetes* **57**, 2603-2612

50. Vilsboll, T. (2009) The effects of glucagon-like peptide-1 on the beta cell. *Diabetes, obesity & metabolism* **11 Suppl 3**, 11-18

51. Bulotta, A., Hui, H., Anastasi, E., Bertolotto, C., Boros, L. G., Di Mario, U., and Perfetti, R. (2002) Cultured pancreatic ductal cells undergo cell cycle re-distribution and beta-cell-like differentiation in response to glucagon-like peptide-1. *Journal of molecular endocrinology* **29**, 347-360

52. Farilla, L., Bulotta, A., Hirshberg, B., Li Calzi, S., Khoury, N., Noushmehr, H., Bertolotto, C., Di Mario, U., Harlan, D. M., and Perfetti, R. (2003) Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. *Endocrinology* **144**, 5149-5158

53. Matsumura, T., Itoh, H., Watanabe, N., Oda, Y., Tanaka, M., Namba, M., Kono, N., Matsuyama, T., Komatsu, R., and Matsuzawa, Y. (1992) Glucagonlike peptide-1(7-36)amide suppresses glucagon secretion and decreases cyclic AMP concentration in cultured In-R1-G9 cells. *Biochemical and biophysical research communications* **186**, 503-508

54. Goke, R., Wagner, B., Fehmann, H. C., and Goke, B. (1993) Glucose-dependency of the insulin stimulatory effect of glucagon-like peptide-1 (7-36) amide on the rat pancreas. *Research in experimental medicine. Zeitschrift fur die gesamte experimentelle Medizin einschliesslich experimenteller Chirurgie* **193**, 97-103

55. Montrose-Rafizadeh, C., Egan, J. M., and Roth, J. (1994) Incretin hormones regulate glucose-dependent insulin secretion in RIN 1046-38 cells: mechanisms of action. *Endocrinology* **135**, 589-594

56. Vahl, T. P., Tauchi, M., Durler, T. S., Elfers, E. E., Fernandes, T. M., Bitner, R. D., Ellis, K. S., Woods, S. C., Seeley, R. J., Herman, J. P., and D'Alessio, D. A. (2007) Glucagon-like peptide-1 (GLP-1) receptors expressed on nerve terminals in the portal vein mediate the effects of endogenous GLP-1 on glucose tolerance in rats. *Endocrinology* **148**, 4965-4973

57. Chang, A. M., Jakobsen, G., Sturis, J., Smith, M. J., Bloem, C. J., An, B., Galecki, A., and Halter, J. B. (2003) The GLP-1 derivative NN2211 restores beta-cell sensitivity to glucose in type 2 diabetic patients after a single dose. *Diabetes* **52**, 1786-1791

58. Fehmann, H. C., and Habener, J. F. (1992) Insulinotropic hormone glucagon-like peptide-I(7-37) stimulation of proinsulin gene expression and proinsulin biosynthesis in insulinoma beta TC-1 cells. *Endocrinology* **130**, 159-166

59. Rask, E., Olsson, T., Soderberg, S., Johnson, O., Seckl, J., Holst, J. J., Ahren, B., Northern Sweden Monitoring of, T., and Determinants in Cardiovascular, D. (2001) Impaired incretin response after a mixed meal is associated with insulin resistance in nondiabetic men. *Diabetes care* **24**, 1640-1645

60.   Lim, G. E., and Brubaker, P. L. (2006) Glucagon-like peptide 1 secretion by the L-cell - The view from within. *Diabetes* **55**, S70-S77

61.   Gribble, F. M., Williams, L., Simpson, A. K., and Reimann, F. (2003) A novel glucose-sensing mechanism contributing to glucagon-like peptide-1 secretion from the GLUTag cell line. *Diabetes* **52**, 1147-1154

62.   Antinozzi, P. A., Ishihara, H., Newgard, C. B., and Wollheim, C. B. (2002) Mitochondrial metabolism sets the maximal limit of fuel-stimulated insulin secretion in a model pancreatic beta cell: a survey of four fuel secretagogues. *J Biol Chem* **277**, 11746-11755

63.   Brand, M. D., and Nicholls, D. G. (2011) Assessing mitochondrial dysfunction in cells. *Biochem J* **435**, 297-312

64.   Lu, D. H., Mulder, H., Zhao, P. Y., Burgess, S. C., Jensen, M. V., Kamzolova, S., Newgard, C. B., and Sherry, A. D. (2002) C-13 NMR isotopomer analysis reveals a connection between pyruvate cycling and glucose-stimulated insulin secretion (GSIS). *P Natl Acad Sci USA* **99**, 2708-2713

65.   Fransson, U., Rosengren, A. H., Schuit, F. C., Renstrom, E., and Mulder, H. (2006) Anaplerosis via pyruvate carboxylase is required for the fuel-induced rise in the ATP : ADP ratio in rat pancreatic islets. *Diabetologia* **49**, 1578-1586

66.   Henquin, J. C., Dufrane, D., and Nenquin, M. (2006) Nutrient control of insulin secretion in isolated normal human islets. *Diabetes* **55**, 3470-3477

67.   Frigerio, F., Casimir, M., Carobbio, S., and Maechler, P. (2008) Tissue specificity of mitochondrial glutamate pathways and the control of metabolic homeostasis. *Biochimica et biophysica acta* **1777**, 965-972

68.   Carobbio, S., Ishihara, H., Fernandez-Pascual, S., Bartley, C., Martin-Del-Rio, R., and Maechler, P. (2004) Insulin secretion profiles are modified by overexpression of glutamate dehydrogenase in pancreatic islets. *Diabetologia* **47**, 266-276

69.   Roduit, R., Nolan, C., Alarcon, C., Moore, P., Barbeau, A., Delghingaro-Augusto, V., Przybykowski, E., Morin, J., Masse, F., Massie, B., Ruderman, N., Rhodes, C., Poitout, V., and Prentki, M. (2004) A role for the malonyl-CoA/long-chain acyl-CoA pathway of lipid signaling in the regulation of insulin secretion in response to both fuel and nonfuel stimuli. *Diabetes* **53**, 1007-1019

70.   Guay, C., Madiraju, S. R., Aumais, A., Joly, E., and Prentki, M. (2007) A role for ATP-citrate lyase, malic enzyme, and pyruvate/citrate cycling in glucose-induced insulin secretion. *J Biol Chem* **282**, 35657-35665

71.   Pongratz, R. L., Kibbey, R. G., Shulman, G. I., and Cline, G. W. (2007) Cytosolic and mitochondrial malic enzyme isoforms differentially control insulin secretion. *J Biol Chem* **282**, 200-207

72.   Ronnebaum, S. M., Jensen, M. V., Hohmeier, H. E., Burgess, S. C., Zhou, Y. P., Qian, S., MacNeil, D., Howard, A., Thornberry, N., Ilkayeva, O.,

Lu, D., Sherry, A. D., and Newgard, C. B. (2008) Silencing of cytosolic or mitochondrial isoforms of malic enzyme has no effect on glucose-stimulated insulin secretion from rodent islets. *J Biol Chem* **283**, 28909-28917

73. Rubi, B., del Arco, A., Bartley, C., Satrustegui, J., and Maechler, P. (2004) The malate-aspartate NADH shuttle member Aralar1 determines glucose metabolic fate, mitochondrial activity, and insulin secretion in beta cells. *J Biol Chem* **279**, 55659-55666

74. Bender, K., Maechler, P., McClenaghan, N. H., Flatt, P. R., and Newsholme, P. (2009) Overexpression of the malate-aspartate NADH shuttle member Aralar1 in the clonal beta-cell line BRIN-BD11 enhances amino-acid-stimulated insulin secretion and cell metabolism. *Clinical science* **117**, 321-330

75. Ronnebaum, S. M., Ilkayeva, O., Burgess, S. C., Joseph, J. W., Lu, D., Stevens, R. D., Becker, T. C., Sherry, A. D., Newgard, C. B., and Jensen, M. V. (2006) A pyruvate cycling pathway involving cytosolic NADP-dependent isocitrate dehydrogenase regulates glucose-stimulated insulin secretion. *J Biol Chem* **281**, 30593-30602

76. Ivarsson, R., Quintens, R., Dejonghe, S., Tsukamoto, K., in 't Veld, P., Renstrom, E., and Schuit, F. C. (2005) Redox control of exocytosis: regulatory role of NADPH, thioredoxin, and glutaredoxin. *Diabetes* **54**, 2132-2142

77. Reinbothe, T. M., Ivarsson, R., Li, D. Q., Niazi, O., Jing, X., Zhang, E., Stenson, L., Bryborn, U., and Renstrom, E. (2009) Glutaredoxin-1 mediates NADPH-dependent stimulation of calcium-dependent insulin secretion. *Mol Endocrinol* **23**, 893-900

78. Schuit, F., De Vos, A., Farfari, S., Moens, K., Pipeleers, D., Brun, T., and Prentki, M. (1997) Metabolic fate of glucose in purified islet cells. Glucose-regulated anaplerosis in beta cells. *J Biol Chem* **272**, 18572-18579

79. Patterson, G. H., Knobel, S. M., Arkhammar, P., Thastrup, O., and Piston, D. W. (2000) Separation of the glucose-stimulated cytoplasmic and mitochondrial NAD(P)H responses in pancreatic islet beta cells. *Proc Natl Acad Sci U S A* **97**, 5203-5207

80. Spegel, P., Sharoyko, V. V., Gohring, I., Danielsson, A. P., Malmgren, S., Nagorny, C. L., Andersson, L. E., Koeck, T., Sharp, G. W., Straub, S. G., Wollheim, C. B., and Mulder, H. (2013) Time-resolved metabolomics analysis of ss-cells implicates the pentose phosphate pathway in the control of insulin release. *Biochem J*

81. Goehring, I., Sauter, N. S., Catchpole, G., Assmann, A., Shu, L., Zien, K. S., Moehlig, M., Pfeiffer, A. F., Oberholzer, J., Willmitzer, L., Spranger, J., and Maedler, K. (2011) Identification of an intracellular metabolic

signature impairing beta cell function in the rat beta cell line INS-1E and human islets. *Diabetologia* **54**, 2584-2594

82. Rebelato, E., Abdulkader, F., Curi, R., and Carpinelli, A. R. (2011) Control of the intracellular redox state by glucose participates in the insulin secretion mechanism. *PloS one* **6**, e24507

83. Khatra, B. S., Chiasson, J. L., Shikama, H., Exton, J. H., and Soderling, T. R. (1980) Effect of Epinephrine and Insulin on the Phosphorylation of Phosphorylase-Phosphatase Inhibitor-1 in Perfused Rat Skeletal-Muscle. *Febs Lett* **114**, 253-256

84. Dietz, M. R., Chiasson, J. L., Soderling, T. R., and Exton, J. H. (1980) Epinephrine Regulation of Skeletal-Muscle Glycogen-Metabolism - Studies Utilizing the Perfused Rat Hindlimb Preparation. *Journal of Biological Chemistry* **255**, 2301-2307

85. Chiasson, J. L., Dietz, M. R., Shikama, H., Wootten, M., and Exton, J. H. (1980) Insulin Regulation of Skeletal-Muscle Glycogen-Metabolism. *American Journal of Physiology* **239**, E69-E74

86. Chiasson, J. L., Atkinson, R. L., Cherrington, A. D., Keller, U., Sinclairsmith, B. C., Lacy, W. W., and Liljenquist, J. E. (1980) Effects of Insulin at 2 Dose Levels on Gluconeogenesis from Alanine in Fasting Man. *Metabolism* **29**, 810-818

87. S. Falkmer, B. H., I.-B. Täljdal. (1970) The structure and metabolism of the pancreatic islets. *Wenner-Gren Center International Symposium Series, Pergamon Press Inc* **16**

88. Toreson, W. E. (1951) Glycogen infiltration (so-called hydropic degeneration) in the pancreas in human and experimental diabetes mellitus. *The American journal of pathology* **27**, 327-347

89. Williamson, J. R. (1960) Electron microscopy of glycogenic changes in beta cells in experimental diabetes. *Diabetes* **9**, 471-480

90. Volk, B. W., and Lazarus, S. S. (1958) The Effect of Various Diabetogenic Hormones on the Structure of the Rabbit Pancreas. *American Journal of Pathology* **34**, 121-135

91. Graf, R., and Klessen, C. (1981) Glycogen in pancreatic islets of steroid diabetic rats. Carbohydrate histochemical detection and localization using an immunocytochemical technique. *Histochemistry* **73**, 225-232

92. Tuomi, T., Santoro, N., Caprio, S., Cai, M., Weng, J., and Groop, L. (2014) The many faces of diabetes: a disease with increasing heterogeneity. *Lancet* **383**, 1084-1094

93. Groop, L., Forsblom, C., Lehtovirta, M., Tuomi, T., Karanko, S., Nissen, M., Ehrnstrom, B. O., Forsen, B., Isomaa, B., Snickars, B., and Taskinen, M. R. (1996) Metabolic consequences of a family history of NIDDM (the Botnia study): evidence for sex-specific parental effects. *Diabetes* **45**, 1585-1593

94. Perley, M. J., and Kipnis, D. M. (1967) Plasma Insulin Responses to Oral and Intravenous Glucose - Studies in Normal and Diabetic Subjects. *Journal of Clinical Investigation* **46**, 1954-&

95. Bagdade, J. D., Bierman, E. L., and Porte, D. (1967) Significance of Basal Insulin Levels in Evaluation of Insulin Response to Glucose in Diabetic and Nondiabetic Subjects. *Journal of Clinical Investigation* **46**, 1549-&

96. Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A., and Butler, P. C. (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* **52**, 102-110

97. Kloppel, G., Lohr, M., Habich, K., Oberholzer, M., and Heitz, P. U. (1985) Islet Pathology and the Pathogenesis of Type-1 and Type-2 Diabetes-Mellitus Revisited. *Surv Syn Pathol Res* **4**, 110-125

98. Tuomi, T., Groop, L. C., Zimmet, P. Z., Rowley, M. J., Knowles, W., and Mackay, I. R. (1993) Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. *Diabetes* **42**, 359-362

99. Fajans, S. S., Bell, G. I., Bowden, D. W., Halter, J. B., and Polonsky, K. S. (1994) Maturity-Onset Diabetes of the Young. *Life Sci* **55**, 413-422

100. Owen, K., and Hattersley, A. T. (2001) Maturity-onset diabetes of the young: from clinical description to molecular genetic characterization. *Best practice & research. Clinical endocrinology & metabolism* **15**, 309-323

101. International_Diabetes_Federation. (2013) IDF Diabetes Atlas. 6th Ed., Brussels

102. Unger, R. H., and Cherrington, A. D. (2012) Glucagonocentric restructuring of diabetes: a pathophysiologic and therapeutic makeover. *J Clin Invest* **122**, 4-12

103. Butler, P. C., and Rizza, R. A. (1991) Contribution to postprandial hyperglycemia and effect on initial splanchnic glucose clearance of hepatic glucose cycling in glucose-intolerant or NIDDM patients. *Diabetes* **40**, 73-81

104. D'Alessio, D. (2011) The role of dysregulated glucagon secretion in type 2 diabetes. *Diabetes, obesity & metabolism* **13 Suppl 1**, 126-132

105. Lee, Y., Wang, M. Y., Du, X. Q., Charron, M. J., and Unger, R. H. (2011) Glucagon receptor knockout prevents insulin-deficient type 1 diabetes in mice. *Diabetes* **60**, 391-397

106. Toft-Nielsen, M. B., Damholt, M. B., Madsbad, S., Hilsted, L. M., Hughes, T. E., Michelsen, B. K., and Holst, J. J. (2001) Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic patients. *The Journal of clinical endocrinology and metabolism* **86**, 3717-3723

107. Vilsboll, T., Agerso, H., Krarup, T., and Holst, J. J. (2003) Similar elimination rates of glucagon-like peptide-1 in obese type 2 diabetic

patients and healthy subjects. *The Journal of clinical endocrinology and metabolism* **88**, 220-224

108. Mannucci, E., Ognibene, A., Cremasco, F., Bardini, G., Mencucci, A., Pierazzuoli, E., Ciani, S., Fanelli, A., Messeri, G., and Rotella, C. M. (2000) Glucagon-like peptide (GLP)-1 and leptin concentrations in obese patients with Type 2 diabetes mellitus. *Diabetic medicine : a journal of the British Diabetic Association* **17**, 713-719

109. Nauck, M. A., Vardarli, I., Deacon, C. F., Holst, J. J., and Meier, J. J. (2011) Secretion of glucagon-like peptide-1 (GLP-1) in type 2 diabetes: what is up, what is down? *Diabetologia* **54**, 10-18

110. Lim, E. L., Hollingsworth, K. G., Aribisala, B. S., Chen, M. J., Mathers, J. C., and Taylor, R. (2011) Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol. *Diabetologia* **54**, 2506-2514

111. Johansson, K., Neovius, M., and Hemmingsson, E. (2014) Effects of anti-obesity drugs, diet, and exercise on weight-loss maintenance after a very-low-calorie diet or low-calorie diet: a systematic review and meta-analysis of randomized controlled trials. *The American journal of clinical nutrition* **99**, 14-23

112. Gloy, V. L., Briel, M., Bhatt, D. L., Kashyap, S. R., Schauer, P. R., Mingrone, G., Bucher, H. C., and Nordmann, A. J. (2013) Bariatric surgery versus non-surgical treatment for obesity: a systematic review and meta-analysis of randomised controlled trials. *Bmj-Brit Med J* **347**

113. Hohmeier, H. E., Mulder, H., Chen, G., Henkel-Rieger, R., Prentki, M., and Newgard, C. B. (2000) Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* **49**, 424-430

114. Ravassard, P., Hazhouz, Y., Pechberty, S., Bricout-Neveu, E., Armanet, M., Czernichow, P., and Scharfmann, R. (2011) A genetically engineered human pancreatic beta cell line exhibiting glucose-inducible insulin secretion. *J Clin Invest* **121**, 3589-3597

115. Powers, A. C., Efrat, S., Mojsov, S., Spector, D., Habener, J. F., and Hanahan, D. (1990) Proglucagon processing similar to normal islets in pancreatic alpha-like cell line derived from transgenic mouse tumor. *Diabetes* **39**, 406-414

116. Lee, Y. C., Asa, S. L., and Drucker, D. J. (1992) Glucagon gene 5'-flanking sequences direct expression of simian virus 40 large T antigen to the intestine, producing carcinoma of the large bowel in transgenic mice. *J Biol Chem* **267**, 10705-10708

117. Hamaguchi, K., and Leiter, E. H. (1990) Comparison of cytokine effects on mouse pancreatic alpha-cell and beta-cell lines. Viability, secretory function, and MHC antigen expression. *Diabetes* **39**, 415-425

118. Drucker, D. J., Jin, T., Asa, S. L., Young, T. A., and Brubaker, P. L. (1994) Activation of proglucagon gene transcription by protein kinase-A in a novel mouse enteroendocrine cell line. *Mol Endocrinol* **8**, 1646-1655

119. Hopcroft, D. W., Mason, D. R., and Scott, R. S. (1985) Insulin secretion from perifused rat pancreatic pseudoislets. *In vitro cellular & developmental biology : journal of the Tissue Culture Association* **21**, 421-427

120. Hopcroft, D. W., Mason, D. R., and Scott, R. S. (1985) Structure-function relationships in pancreatic islets: support for intraislet modulation of insulin secretion. *Endocrinology* **117**, 2073-2080

121. Meda, P., Chanson, M., Pepper, M., Giordano, E., Bosco, D., Traub, O., Willecke, K., el Aoumari, A., Gros, D., Beyer, E. C., and et al. (1991) In vivo modulation of connexin 43 gene expression and junctional coupling of pancreatic B-cells. *Experimental cell research* **192**, 469-480

122. Hauge-Evans, A. C., Squires, P. E., Persaud, S. J., and Jones, P. M. (1999) Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca2+ and insulin secretory responses of MIN6 pseudoislets. *Diabetes* **48**, 1402-1408

123. Hannon, G. J. (2002) RNA interference. *Nature* **418**, 244-251

124. Danielsson, A. P., Moritz, T., Mulder, H., and Spegel, P. (2010) Development and optimization of a metabolomic method for analysis of adherent cell cultures. *Anal Biochem* **404**, 30-39

125. Saito, N., Ohashi, Y., Soga, T., and Tomita, M. (2010) Unveiling cellular biochemical reactions via metabolomics-driven approaches. *Current opinion in microbiology* **13**, 358-362

126. Spegel, P., Malmgren, S., Sharoyko, V. V., Newsholme, P., Koeck, T., and Mulder, H. (2011) Metabolomic analyses reveal profound differences in glycolytic and tricarboxylic acid cycle metabolism in glucose-responsive and -unresponsive clonal beta-cell lines. *Biochem J* **435**, 277-284

127. Stamenkovic, J. A., Andersson, L. E., Adriaenssens, A. E., Bagge, A., Sharoyko, V. V., Gribble, F., Reimann, F., Wollheim, C. B., Mulder, H., and Spegel, P. (2015) Inhibition of the malate-aspartate shuttle in mouse pancreatic islets abolishes glucagon secretion without affecting insulin secretion. *Biochem J* **468**, 49-63

128. Madsen, R., Lundstedt, T., and Trygg, J. (2010) Chemometrics in metabolomics--a review in human disease diagnosis. *Analytica chimica acta* **659**, 23-33

129. Trygg, J., and Wold, S. (2002) Orthogonal projections to latent structures (O-PLS). *J Chemometr* **16**, 119-128

130. Wiklund, S., Johansson, E., Sjostrom, L., Mellerowicz, E. J., Edlund, U., Shockcor, J. P., Gottfries, J., Moritz, T., and Trygg, J. (2008) Visualization of GC/TOF-MS-based metabolomics data for identification

of biochemically interesting compounds using OPLS class models. *Anal Chem* **80**, 115-122

131. Efron, B., and Gong, G. (1983) A Leisurely Look at the Bootstrap, the Jackknife, and Cross-Validation. *Am Stat* **37**, 36-48

132. Piper, K., Brickwood, S., Turnpenny, L. W., Cameron, I. T., Ball, S. G., Wilson, D. I., and Hanley, N. A. (2004) Beta cell differentiation during early human pancreas development. *The Journal of endocrinology* **181**, 11-23

133. Blodgett, D. M., Nowosielska, A., Afik, S., Pechhold, S., Cura, A. J., Kennedy, N. J., Kim, S., Kucukural, A., Davis, R. J., Kent, S. C., Greiner, D. L., Garber, M. G., Harlan, D. M., and diIorio, P. (2015) Novel Observations From Next-Generation RNA Sequencing of Highly Purified Human Adult and Fetal Islet Cell Subsets. *Diabetes* **64**, 3172-3181

134. Ahn, Y. B., Xu, G., Marselli, L., Toschi, E., Sharma, A., Bonner-Weir, S., Sgroi, D. C., and Weir, G. C. (2007) Changes in gene expression in beta cells after islet isolation and transplantation using laser-capture microdissection. *Diabetologia* **50**, 334-342

135. Giuliani, M., Moritz, W., Bodmer, E., Dindo, D., Kugelmeier, P., Lehmann, R., Gassmann, M., Groscurth, P., and Weber, M. (2005) Central necrosis in isolated hypoxic human pancreatic islets: evidence for postisolation ischemia. *Cell transplantation* **14**, 67-76

136. Olsen, H. L., Theander, S., Bokvist, K., Buschard, K., Wollheim, C. B., and Gromada, J. (2005) Glucose stimulates glucagon release in single rat alpha-cells by mechanisms that mirror the stimulus-secretion coupling in beta-cells. *Endocrinology* **146**, 4861-4870

137. Le Marchand, S. J., and Piston, D. W. (2010) Glucose suppression of glucagon secretion: metabolic and calcium responses from alpha-cells in intact mouse pancreatic islets. *J Biol Chem* **285**, 14389-14398

138. Franklin, I., Gromada, J., Gjinovci, A., Theander, S., and Wollheim, C. B. (2005) beta-Cell secretory products activate alpha-cell ATP-dependent potassium channels to inhibit glucagon release. *Diabetes* **54**, 1808-1815

139. Powell, D. R., DaCosta, C. M., Gay, J., Ding, Z. M., Smith, M., Greer, J., Doree, D., Jeter-Jones, S., Mseeh, F., Rodriguez, L. A., Harris, A., Buhring, L., Platt, K. A., Vogel, P., Brommage, R., Shadoan, M. K., Sands, A. T., and Zambrowicz, B. (2013) Improved glycemic control in mice lacking Sglt1 and Sglt2. *American journal of physiology. Endocrinology and metabolism* **304**, E117-130