Shared Metabolic Pathways in Fuel-Stimulated Insulin Secretion

by

Matthew Lester Odegaard

Department of Pharmacology and Cancer Biology
Duke University

Date:_______________________
Approved:

____
Christopher Newgard, Supervisor

____
Anthony Means

____
Deborah Muoio

____
Marc Caron

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Pharmacology and Cancer Biology in the Graduate School
of Duke University

2009

ABSTRACT

Shared Metabolic Pathways in Fuel-Stimulated Insulin Secretion

by

Matthew Lester Odegaard

Department of Pharmacology and Cancer Biology
Duke University

Date:_______________________
Approved:

____
Chris Newgard, Supervisor

____
Anthony Means

____
Deborah Muoio

____
Marc Caron

An abstract of a dissertation submitted in partial
fulfillment of the requirements for the degree
of Doctor of Philosophy in the Department of
Pharmacology and Cancer Biology in the Graduate School
of Duke University

2009

Abstract

Insulin secretion is a fundamental process of pancreatic β-cells required for the maintenance of glucose homeostasis. Fuel-stimulated insulin secretion occurs in proportion to the rate of metabolism of fuel substrates, yet the signals generated by metabolism of these secretagogues are incompletely understood. The increased burden placed on the β-cell in conditions of obesity and insulin resistance often leads to dysregulation of stimulous-secretion coupling. Therefore, better understanding of the metabolic events required for insulin release is likely to be helpful in development of more effective treatments for diabetes.

Previous work in our lab revealed a critical role for the pyruvate-isocitrate cycling pathway in glucose-stimulated insulin secretion. It has been our hypothesis that this series of reactions plays a unique role in the β-cell, and may be responsible for the generation of second-messenger signals critical for insulin secretion in response to increased fuel metabolism. One of the intermediates in the pyruvate/isocitrate cycle is cytosolic 2-oxoglutarate (2OG). In an effort to better understand the components of the pyruvate-isocitrate cycle and the signals that it generates, we initially focused our studies on the transporter protein responsible for the return of 2OG to the mitochondria, the 2-oxoglutarate carrier (OGC).

OGC was overexpressed and suppressed in both rat insulinoma 832/13 β-cells and islets, and effects on metabolism and insulin secretion were measured. While overexpression of the OGC failed to alter insulin secretion, its siRNA-mediated suppression resulted in decreased insulin secretion in response to glucose, glutamine + BCH, and dimethyl-2-oxoglutarate. Suppression of OGC did not affect core pathways of fuel metabolism such as glucose usage, glucose oxidation or ATP production during glucose-stimulated insulin secretion (GSIS) or glutamine oxidation or ATP production during amino acid-stimulated insulin secretion (AASIS). Similar to previous findings, glucose-induced NADPH production was determined to be decreased in response to OGC suppression, whereas NADPH production during AASIS in untreated cells was already much lower than for GSIS, and suppression of OGC failed to decrease NADPH further.

As an additional approach to studying the role of 2OG metabolism in insulin secretion, we also investigated the mitochondrial enzyme glutamate dehydrogenase (Glud1). Overexpression of wild-type Glud1 failed to alter insulin secretion in 832/13 cells or in islets; however, suppression of Glud1 decreased both GSIS and AASIS, but did not affect dimethyl-2OG-stimulated insulin secretion. The reduction in AASIS was most likely the result of reduced glutamine oxidation. In contrast, during GSIS, NADPH production was decreased by Glud1 suppression, similar to our observation with the OGC.

In summary, these data expand our understanding of the metabolic pathways necessary for insulin secretion, and support the idea of a common metabolic pathway required for fuel-stimulated insulin release, including flux through the OGC, Glud1, and ICDc. However, while these data support the hypothesis that NADPH production is necessary for robust GSIS, it plays a less-prominent role during AASIS, and most likely works in concert with additional coupling-factors and signals.

Contents

List of Tables

List of Figures

List of Abbreviations

2OG, 2-oxoglutarate

AASIS, amino acid- (glutamine) stimulated insulin secretion

AdCMV-βGAL, adenovirus overexpressing β-galactosidase (control)

AdCMV-Glud1, adenovirus overexpressing wild-type glutamate dehydrogenase

AdCMV-OGC, adenovirus overexpressing the 2-oxoglutarate carrier

Ad-siControl, adenovirus containing a non-homologous siRNA sequence (control)

Ad-siGlud1-1, adenovirus containing an siRNA sequence targeting Glud1

Ad-siGlud1-2, adenovirus containing an siRNA sequence targeting Glud1

Ad-siOGC#1, adenovirus containing an siRNA sequence targeting the OGC

Ad-siOGC#2, adenovirus containing an siRNA sequence targeting the OGC

asp*, asparagine/ aspartate

BCH, 2-amino-2-norbornanecarboxylic acid

CIC, citrate/ isocitrate carrier

CL, citrase lyase

DIC, dicarboxylate carrier

DMM, dimethyl malate

dm-2OG, dimethyl-2-oxoglutarate

EGCG, epigallocatechin gallate

Glud1, glutamate dehydrogenase

glu*, glutamine/ glutamate

GSIS, glucose-stimulated insulin secretion

ICDc, isocitrate dehydrogenase, NADP-dependent cytosolic form

K_{ATP}, ATP-sensitive potassium channel

KIC, ketoisocaproate

LC-CoA, long-chain coenzyme-A

OAA, oxaloacetate

OGC, 2-oxoglutarate carrier

PAA, phenyl acetic acid

PC, pyruvate carboxylate

PDH, pyruvate dehydrogenase

RIA, radio immuno-assay

ss-2OG, sodium-salt of 2-oxoglutarate

SUR1, sulfonylurea receptor 1

TCA, tricarboxylic acid

Acknowledgements

In many ways, working towards a graduate research degree is a mix of extremes of personal growth and frustration. Making it through such times mentally-intact would not be possible without the help of coworkers, friends and family, all of whom deserve a great deal of thanks.

I would like to start by thanking my advisor and mentor, Dr. Chris Newgard. I feel extremely fortunate to have received training in his lab, and for the past several years have had a virtual a front-row seat of sorts to not only cutting-edge advances in β-cell biology and diabetes, but also new trends in approaches to scientific research projects. Chris has fostered a strong atmosphere of both internal and external cooperation, ultimately stemming from the high value he places on people; this emphasis on cooperation combines the strengths of many different individuals and institutions to synergize research progress, and has been an incredible benefit to my own personal scientific training. I greatly admire Chris's ability to manage multiple diverse projects across numerous institutions, and his passion and dedication to investigating a disease that affects so many people.

I would also like to specially thank Dr. Jamie Joseph, who spent more than a year working very closely with me at the bench. Jamie's generosity in getting me started with a hypothesis-driven research project ultimately made all the difference in my graduate school experience. And without his direct and extensive help, I would have had great difficulty overcoming many of the trouble-shooting issues necessary to get the project off of the ground. His patience in mentoring and rigor and skill as a scientist have taught me a great deal about molecular biology research.

Dr. Mette Jensen was one of the first people I worked with in the lab, during my second rotation in the Pharmacology Department. Over the past several years I have relied on her for scientific insight into various pieces of data, and am very grateful to her for all her help with experiments.

Dr. Thomas Becker has been another source of great help over the years. Beyond the science though, we also share a number of unique outside interests, and I have enjoyed our conversations and outside pursuits.

In the summer of 2008, I had a chance to be a project SEED mentor, working with a summer student in the lab, Charles Ramsey, from NCSSM. Dr. Michelle Arlotto was the driving force behind our lab's involvement in project SEED, and I am indebted to her for all the help she provided with the program, and her motivation in making the opportunity available. I have enjoyed our conversations since then, and appreciate her scientific support and encouragement.

Beyond these individuals, I also owe thanks to many others in the lab, including past and present postdocs and students, and the research scientists who make up the foundation of the lab environment. In our immediate group Dr. Hans Hohmeier and Dr.

Danhong Lu, among others, have given me a great deal of help over the years, and I have greatly appreciated both the company and technical expertise of Dr. Tim Koves, April Hawkins, and others in Dr. Deb Muoio's metabolism group. I have also greatly enjoyed the numerous conversations I've had with Dr. Igor Nepliouev, covering topics ranging from bread making to economics.

I want to also thank my family. From the time I was very young, my parents, Les and Jan Odegaard, helped me develop a very strong interest in biology, in one form or another, and I am grateful to have been given so much opportunity in that area. My brother Brian and sister Laura, whether close to home, or thousands of miles away across the U.S., always gave me their support and encouragement, even as they balanced their own education and training. And my father-in-law Tom Hanks Sr., late mother-in-law Debbie, brother-in-law Tom and sister-in-law Nancy, have been absolutely wonderful; I could not have married into a nicer family.

Finally, and most importantly, I want to thank my wife Mary, and daughter Kalina, whose smiles will always make me melt. Mary has been there for me from the very beginning, and has had to put so much trust in me from day one of moving to North Carolina. Her kindness and patience were invaluable throughout a process that she had so little control over. And her hard work has done, and continues to do, so much for us as a family. I am a very lucky man to be married to such an incredible woman.

1. Introduction

Glucose homeostasis in the blood is achieved through a complex whole-body regulatory system involving glucose-sensing by the endocrine pancreas coupled to changes in glucose metabolism by peripheral tissues such as muscle, fat, and liver. In the fasted state, glucose levels in healthy individuals should be maintained below 100 mg/dl (5.5mM) (1). However, because glucose is the obligate metabolic fuel for the brain under physiologic conditions (2), it is critical that plasma levels not drop below ~3 mM, as functional brain failure (seizure, coma) and death can potentially result (3). Thus, the body has evolved a multitude of systems to prevent this from occurring, including glucagon secretion from the pancreas, such that hypoglycemia in the absence of insulin injection is an uncommon clinical event (4). In contrast, in the fed (postprandial) state, an acute increase in plasma glucose levels triggers a strong insulin secretory response, rapidly bringing glucose levels under conrol. Dysregulated hormone secretion from the pancreas leads to loss of glycemic control and overt diabetes (5).

1.1 Islets of Langerhans

The structures that make up the endocrine pancreas and are responsible for glucose-sensing and regulation are the islets of Langerhans, first described by Paul Langerhans in 1869 (6), but established by Banting and Best to be the structures responsible for the secretion of the hormone insulin. Banting and Best were also the first to show that insulin was able to reverse hyperglycemia in rabbits, leading to its use in treatment of juvenile diabetes (7; 8). Islets are approximately 150uM in size (9), number ~2 million within the human pancreas (10), and are made up of several different cell types, each responsible for secreting a different hormone. Delta cells secrete somatostatin (11), epsilon cells secrete ghrelin (12), and PP cells secrete pancreatic polypeptide (13). The two most abundant cell types in the islet are the α-cells and β-cells. α-cells are found in the periphery of the islet, and under fasting conditions secrete the hormone glucagon, which opposes the actions of insulin by mobilizing glucose stores in liver (14). In contrast, a few dozen to a few thousand β-cells make up the vast majority of the core of each islet (15) and are responsible for secreting the hormone insulin, which under normal conditions in the fed state is responsible for suppressing glucagon release, facilitating plasma glucose uptake into muscle and fat and suppressing gluconeogenesis in the liver (16).

While β-cells are exquisitely fine-tuned for their role in glucose sensing, they are also a relatively fragile cell type. A number of genetic factors and metabolic perturbations can lead to β-cell dysfunction or death, resulting in unregulated blood glucose and diabetes. In type 1 diabetes, autoimmune destruction of β-cells renders an individual unable to produce their own insulin; type 1 diabetics must therefore manage their plasma glucose levels by injection of insulin. Two rare forms of monogenic diabetes, maturity-onset diabetes of the young (MODY) and permanent neonatal diabetes mellitus (PNDM), are thought to be cause by mutations in single genes, leading to both insulin-dependent and insulin-resistant diabetic outcomes (17). Gestational diabetes affects ~14% of all pregnant women, and is caused by increased insulin resistance in the mother, potentially foreshadowing the development of type 2 diabetes later in life (18). Finally, type 2 diabetes is closely linked to increased insulin resistance in the peripheral tissues and liver, often within the context of patient obesity and excess visceral abdominal fat.

1.2 Pathogenesis of Type 2 Diabetes

Advances in agriculture and food distribution systems have virtually eliminated hunger from modern societies, but at the same time allowed for the ingestion of carbohydrate- and fat-rich food on an almost continual basis. This situation has led to a drastic increase in the rate of obesity, which is one of a group of physiologic risk factors known as the "metabolic syndrome" shown to correlate with increased occurrence of heart disease and type 2 diabetes. Indeed, there is a close relationship between these two diseases; many of the risk factors for coronary artery disease (CAD) are metabolic (19), and as the incidence of obesity over the past few decades has increased at epidemic rates, so too has the incidence of diabetes. Currently, more than a third of adults 20 years or older in the US are considered obese, and approximately 24 million people are thought to have diabetes, with 95% of these cases being type 2 diabetes (20).

In contrast to type 1 diabetes, a hallmark trait of type 2 diabetes is insulin resistance, which limits the therapeutic effect that insulin can have on facilitating glucose uptake in the peripheral tissues and limiting glucose production by the liver. During the course of the disease, as insulin resistance in these tissues gradually increases, there is an initial increase in β-cell number, such that higher plasma insulin levels are achieved during glucose challenge. Because of this compensatory effect the majority of obese individuals are not diabetic (21). However, as the disease progresses, the β-cells gradually become impaired in their ability to sense glucose and secrete insulin. The loss of normal GSIS marks the transition from the pre-diabetic insulin-resistant state to full-blown type 2 diabetes (22). Over time, β-cell survival also begins to decrease, further exacerbating the problem of maintaining glucose homeostasis.

It is for these reasons that investigation of β-cell function is of primary importance, since better understanding of the mechanisms behind glucose-sensing and insulin secretion would be assumed to facilitate development of treatment strategies that address the functional failure observed during progression of type 2 diabetes (23).

1.3 Glucose-Stimulated Insulin Secretion from Pancreatic β-cells

Within the pancreas, the islets are located immediately adjacent to blood vessels and arteries, allowing them to directly monitor plasma glucose levels (24). Multiple lines of evidence indicate that glucose-sensing by the β-cells within the islets is dependent upon the actual catabolic breakdown of glucose (25), as non-metabolizable forms of glucose fail to stimulate insulin secretion (26; 27). In the postprandial state, as plasma glucose levels rise, glucose enters the β-cells through facilitated transport by the Glut2 transporter, which has a higher Km than the Glut4 transporter (found in muscle and other cell types) and is therefore more sensitive to changes in glucose within the physiologic range (28). Once inside the β-cell, glucose then becomes phosphorylated by glucokinase (hexokinase IV). Glucokinase (GK) has a higher Km for glucose than the other hexokinases and is therefore also more sensitive to changes in glucose concentration within the 5-20mM range. Overexpression of GK in rat islets leads to increased insulin secretion under stimulatory conditions (29), which demonstrates the importance of the GK reaction in glucose-sensing.

Phosphorylation of glucose plays an important role in regulating glucose metabolism by trapping it within the cell and committing it to one of several metabolic fates, including breakdown through glycolysis or entry into the pentose phosphate shunt, which is thought to have very limited flux in beta-cells (30). Breakdown of glucose through glycolysis in the cytosol and subsequent oxidation in the tricarboxylic acid (TCA) cycle within the mitochondrial matrix generates an increase in the ATP to ADP ratio, with additional ATP being generated through the oxidation of reducing equivalents (NADH, $FADH_2$) by the electron transport chain in the mitochondrial inner membrane.

1.3.1 K_{ATP}-channel

Production of ATP is thought to be a critical signal for stimulating insulin release. The increase in the ATP to ADP ratio that occurs in response to an increase in glucose metabolism (Fig. 1) results in closure of ATP-dependent potassium channels (K_{ATP}-channel) on the plasma membrane, which are responsible for K+ ion efflux out of the cell (31). Once closed, positive charge builds up within the cytosol until the cell depolarizes, similar to a neuron (32). Depolarization of the cell results in opening of L-type voltage-dependent calcium channels (33) on the plasma membrane, and subsequent influx of extracellular calcium and release of calcium from internal stores (34). These events in turn culminate with activation of CAM kinases and vesicle-associated SNARE family members, followed by trafficking and partial binding of insulin vesicles to the plasma membrane, and insulin release (35-37).

The K_{ATP}-channel is composed of two protein subunits: a regulatory subunit (SUR1 receptor) and an inward rectifying subunit (Kir6.2) (38; 39). Identification and cloning of the K_{ATP}-channel led to characterization of mutations in these proteins in humans, which have been shown to influence insulin secretion and glycemic control (40; 41). Mice heterozygous for functional Kir6.2 subunit show hypersecretion of insulin (42), while the SUR1 receptor is the site of action of the antidiabetic drugs tolbutamide and glipizide, which act to stimulate insulin release through direct depolarization of the β-cell (43).

Although regulation of GSIS by the K_{ATP}-channel has been firmly established, multiple lines of evidence indicate that that the channel is not the exclusive control mechanism, and that other signals and molecular players are also involved. Indeed, SUR1-/- mice are able to maintain some degree of regulation of insulin release and control of glucose homeostasis, demonstrating intact feeding-stimulated insulin secretion (44). Within that context, glucose can still stimulate a 6-fold change in insulin release in SUR1-/- mice (45), as well as an increase in intracellular Ca2+ (46).

1.3.2 Biphasic Insulin Secretion

Glucose-stimulated insulin secretion (GSIS) was observed several decades ago to occur in two temporal phases (47). Upon initial glucose stimulation there is a rapid increase in the rate of insulin release, which constitutes the “first phase” of GSIS. After 10-15 minutes, insulin release decreases to a moderately-elevated secretion rate, marking entry into the “second phase” of GSIS, which can be sustained for several hours if elevated blood glucose levels persist (47).

The different phases of insulin secretion are thought to be due, in part, to rate limiting steps between different pools of insulin-containing granules within the β-cell (48). Closure of the K_{ATP}-channel triggers the first phase of GSIS, in which the “immediately-releasable” pool of insulin-containing granules is secreted (49). Interestingly, while the β-cell has an abundance of insulin secretory vesicles, it is the pool of newly-synthesized insulin granules that appears to be the first released upon glucose stimulation (50). Only 10% of insulin granules in the β-cell are docked at the plasma membrane in a readily-releasable form (48), so first-phase insulin secretion generally results in the release of less than 1% (~100-150 granules) of the total granules in the cell (51).

In the absence of glucose, direct depolarization of the β-cell with high extracellular concentrations of K+ (30mM KCl) plus diazoxide (which locks the K_{ATP}-channel in the open state, allowing influx of K+) only triggers first-phase insulin secretion (52). However, under these conditions the addition of stimulatory concentrations of glucose still leads to additional significant increases in insulin release (53) that do not involve a further increase in intracellular [Ca^{2+}] (52). Instead, glucose metabolism is thought to generate non-ATP coupling factors (54) that work to either amplify the actions of Ca^{2+} on exocytosis (55) or work in a Ca^{2+}-independent manner (56), to drive the second phase of insulin secretion.

Second-phase insulin secretion involves both docked and readily-releasable insulin granules from the intracellular storage pool, and occurs at a rate of 5-40 granules per minute over a period of hours (51). Ultimately, the amplifying signals that occur during second-phase insulin secretion contribute to as much as 70% of total GSIS, and are independent of the actions of the K_{ATP}-channel (54). The rate-limiting step in second phase secretion is postulated to be the conversion of readily-releasable insulin granules to the state of immediate releasability (48), and, in a glucose-dependent manner, can be altered by a number of agonists, including glucagon-like peptide 1 and free fatty acids, which require a threshold level of glucose (~6mM) for their effects (57). However, the exact identity of the metabolically-generated coupling factors responsible for second phase insulin secretion is still unknown (58), and is clearly a prerequisite for coherent development of diabetes therapies that target β-cell function (25).

1.3.3 K_{ATP}-Independent Signals in GSIS

Based on the fact that a wide range of metabolic events occur once a β-cell undergoes fuel stimulation, it is not surprisingly that numerous factors in addition to ATP have been shown to correlate with changes in glucose metabolism, and have therefore been proposed to function as second messengers in insulin secretion, including cAMP (59; 60), reactive oxygen species (ROS) (61; 62), and TCA cycle intermediates such as succinate (63).

GTP has been proposed as a coupling factor in insulin secretion (64). Produced by mitochondrial GTP succinyl-CoA synthetase (GTP-SCS), GTP makes an interesting candidate molecule because it is presumably generated in a 1:1 ratio with glucose flux through the TCA cycle, and would therefore be very sensitive to changes in glucose metabolism. In that regard, suppression of GTP-SCS impairs the increase in intracellular calcium that occurs during GSIS (64). However, preliminary data from our group indicates that GTP production may actually decrease during GSIS, and in the current absence of any identified molecular targets for GTP in regulation of insulin secretion, more research will be required to firmly establish a role for this nucleotide.

Another signaling model that has attracted much attention is the malonyl-CoA/ LC-CoA hypothesis. Here, fuel-induced increases in malonyl-CoA lead to inhibition of mitochondrial carnitine palmitoyl transferase (CPT1) and β-oxidation, which results in buildup of free fatty acids and long-chain acyl-CoA (LC-CoA) (65). Although LC-CoA does not stimulate insulin secretion in the absence of glucose (57), free fatty acids mimic the K_{ATP}-channel independent actions of glucose (53), and addition of LC-CoA to permeabilized cells stimulates granule exocytosis (66).

These observations have led to the idea that LC-CoA could play a role in FA-induced potentiation of GSIS, possibly via involvement of the G-protein coupled receptor GPR40 (67). Both long and medium chain fatty acids activate the GPR40 receptor (68), which is specifically expressed in the β-cell and has been linked to impaired glucose homeostasis in mice (69). Although GPR40-/- mice have normal glycemic control and insulin secretion in response to glucose, they exhibit markedly reduced fatty acid potentiation of insulin release (70). Futhermore, overexpression of GPR40 in mice leads to increased insulin secretion in the presence of 11mM glucose plus palmitate (71). It is thought that GPR40 signaling through the $G_{\alpha q}$ PLC pathway leads to generation of IP_3, ultimately augmenting insulin secretion by triggering release of calcium stores from the ER (72).

However, despite the evidence of a role for GPR40 signaling in insulin secretion, disruption of LC-CoA generation by acute treatment with the LC-CoA synthetase inhibitor triacsin C did not affect insulin secretion (73). Decreasing malonyl-CoA levels by overexpression of malonyl-CoA decarboxylase (MCD) also did not impair GSIS (74). In this regard, these data indicate it is likely that GPR40 plays more of a role in exogenous nutrient-sensing than in autocrine signaling through endogenous FFA production by the β-cell.

Another factor that has attracted a great deal of attention is the amino acid glutamate (75), which functions as a neurotransmitter in the central nervous system (76). The hypothesis that glutamate serves as a coupling factor in insulin secretion is supported by the observations that glutamate levels increase during GSIS, and that reduction of glutamate levels by overexpression of glutamate decarboxylase leads to impairment of GSIS (77). Furthermore, (discussed in more detail in chapter 4), glutamine, which can be converted into glutamate through the glutaminase enzyme in the cytosol, has been shown to stimulate insulin secretion in the SUR1-/- mouse (78), which was used as a model for non-metabolic triggering of first phase insulin secretion in order to identify regulators of second-phase insulin release.

However, additional experiments have produced conflicting results regarding the role of glutamate as a coupling factor in the β-cell. Although addition of 16.7mM glucose in the presence of a depolarizing concentration of K^+ is able to augment insulin release, addition of dimethyl-glutamate or glutamine to normal rodent islets only weakly increases insulin secretion (79). Instead, insulin release is only increased further by the addition of leucine, which activates glutamate dehydrogenase (80) and allows entry of glutamate into the TCA cycle as 2OG. These data indicate that glutamate alone in the absence of increased fuel metabolism is unable to alter insulin secretion in islets that have both subunits of the K_{ATP}-channel intact, even in the context of β-cell depolarization. Still, in light of the potential involvement of glutamate metabotropic receptors in insulin secretion (81), an independent role for the amino acid in augmenting insulin release cannot currently be ruled out.

To date, no study has conclusively identified the K_{ATP}-independent signal responsible for augmenting the second phase of insulin secretion. At best, only strong correlations have been made between changes in the levels of likely candidate molecules and altered GSIS. Therefore, our approach has been to focus on a set of specific metabolic pathways and linked enzymatic reactions in the β-cell, using a combination of gene manipulation, metabolic flux analysis and static metabolic profiling to investigate their potential involvement in insulin secretion.

1.3.4 Flux through Pyruvate Carboxylase

One of the clues we have as to which metabolic pathways may be important for generating the additional signals necessary for GSIS comes from the observation that β-cells have an unusually high expression of the mitochondrial enzyme pyruvate carboxylase (PC), and that the ratio of pyruvate flux into the TCA cycle through PC versus pyruvate dehydrogenase (PDH) is approximately equal (30; 82; 83). While pyruvate dehydrogenase (PDH) decarboxylates pyruvate for entry into the TCA cycle as the two-carbon compound acetyl-CoA, PC is responsible for the addition of a carboxyl group to pyruvate to form the TCA cycle intermediate oxaloacetate. Flux through PC plays an important role in anaplerosis (replenishment of TCA cycle intermediates) and, in the liver, gluconeogenesis. However, while β-cells have recently been shown to also express one of the other enzymes necessary for gluconeogenesis, PEPCK, they are considered non-gluconeogenic (84), and have low levels of fatty acid synthase expression and rates of lipogenesis (85); therefore, flux through PC is thought to serve an alternative purpose.

Instead, our lab and others have demonstrated that flux through PC and the closely-related process of pyruvate cycling (described in section 1.4) are directly linked to metabolic pathways necessary for glucose-sensing and insulin secretion (23; 86). It has previously been established that ^{13}C-glucose can be used to estimate pyruvate cycling/ anaplerotic substrate oxidation by analyzing the NMR spectra of glutamate isolated from the cells (87). Using this approach, we have studied several immortalized clonal INS-1 β-cell lines (88) with different insulin secretion capabilities (89), and previously showed that pyruvate flux through PC, and not pyruvate oxidation via PDH, was correlated with the capacity for GSIS in variously glucose-responsive INS-1-derived cell lines (90).

Furthermore, directly increasing PC flux using dimethyl malate (DMM), or decreasing flux using the PC inhibitor phenyl acetic acid (PAA), was shown to lead to a corresponding increase or decrease in insulin secretion, respectively, in the most responsive cell line (832/13) (90). In contrast, no relationship between PDH flux and insulin secretion has been observed, as overexpression of PDH kinase or phosphatase were previously reported to change PDH activity without affecting insulin secretion (91).

Surprisingly, suppression of PC with siRNA did not alter insulin secretion; however, this was revealed to be due to compensatory up-regulation in the activity of the remaining PC protein by buildup of acetyl-CoA (92). Instead, inhibition of PC with phenylacetic acid (PAA), which impairs the allosteric regulation of the enzyme by acetyl-CoA (93), was shown to decrease GSIS both in cell lines and islets (90; 94; 95).

Additional evidence for the importance of PC in insulin secretion comes from studies investigating the role of lipids in insulin secretion. Chronic lipid culture results in upregulation of genes involved in beta-oxidation of fatty acids (96), potentially increasing flux of glucose-derived pyruvate through PC via increases in its allosteric activator acetyl-CoA (97). Within this context, lipid-induced impairment of β-cell insulin secretion is associated with impairment in the glucose-induced increment in pyruvate cycling, and impaired insulin secretion could be rescued by the pyruvate cycling substrate DMM (98; 99). Pyruvate cycling and PC activity were also shown to be increased under conditions of chronic hyperglycemia (100) and in insulin resistant pre-diabetic ZDF rats (101), suggesting that increased cycling may be an intrinsic compensatory mechanism for enhancing insulin secretion in the presence of increased insulin resistance.

Together, these observations define a critical role for PC flux and pyruvate cycling in glucose-sensing and insulin secretion in both normal and disease states.

1.4 Metabolic Events Downstream of PC

While flux through PC has been shown to be important for insulin secretion, our working hypothesis is that one or more of the metabolic pathways downstream of PC ultimately generates the key ATP-independent second messenger signal necessary for robust and sustained GSIS. Specific and unresolved questions include the following: first, which specific metabolic reactions are involved in generating secretion coupling factors? And second, what is the nature of the coupling factors and how do they engage with the secretory granules to elicit insulin secretion?

To address these questions, we have focused our studies on a group of metabolic pathways linked to PC flux termed the "pyruvate cycling reactions", which include the pyruvate-malate, pyruvate-citrate, and pyruvate-isocitrate cycling reactions. The first step in these reactions is the efflux of TCA cycle intermediates (malate, citrate, and isocitrate) through various mitochondrial transporter proteins out of the mitochondria and into the cytosol. Because each molecule of pyruvate converted into OAA by PC results in a net gain of two carbons within the mitochondria (four-carbon OAA minus two CO_2 produced during oxidation) and the TCA cycle is not a carbon sink, efflux of intermediates must occur to balance flux through PC. Once in the cytosol, these TCA cycle intermediates can either be reconverted into pyruvate (hence the collective name "pyruvate cycling reactions") or directed to re-enter the mitochondria again through the mitochondrial transporters.

Our approach to studying the role of the pyruvate cycling reactions in the β-cell has centered on altering the activity/ expression of cycling pathway components and observing the corresponding effects on GSIS. These studies have revealed that only one of the three pathways appears to play a critical role in stimulating insulin secretion (23).

1.4.1 Pyruvate-Malate Cycling

The pyruvate-malate cycle (Fig. 2) involves transport of mitochondrial malate into the cytosol through the dicarboxylate carrier (DIC), with direct conversion of malate into pyruvate via the cytosolic form of malic enzyme (ME1, or MEc). Malate levels have been shown to be increased during glucose-stimulation of clonal β-cells (92) or isolated rodent islets (102), and overexpression of malic enzyme in 832/13 cells slightly increases GSIS (103). However, this is likely simply through augmenting anaplerosis in general, as DMM potentiates GSIS in mouse islets lacking MEc, indicating that in this context malate import, not export, into the mitochondria through the DIC is occurring (103). Furthermore, although adenovirus-mediated siRNA suppression of MEc was shown to decrease GSIS in 832/13 β-cells (104), it failed to decrease insulin secretion in isolated rat islets (105). Finally, islets from MOD-/- mice, which lack MEc, exhibit normal GSIS (105).

Taken together, these observations suggest limited involvement of pyruvate-malate cycling in generating signals that regulate GSIS in an in vivo setting. However, it is important to note an alternative role has recently been suggested for this cycle. β-cells, unlike other metabolically-responsive cells such as myocytes or hepatocytes, cannot accommodate increased glycolytic flux by increasing glycogen or lactate production; instead, substrate cycling, such as through the pyruvate-malate cycle, is thought to be necessary for allowing glycolytic and mitochondrial fluxes to increase in direct proportion to circulating levels of fuel secretagogues (106). Within that context, one of the isoforms of mitochondrial malic enzyme, ME2, is thought to interact with the PDH complex (107), making ME2-derived pyruvate a better substrate for PDH, and potentially enhancing the use of glutamine as a respiratory fuel by shunting glutamate-derived malate towards the formation of pyruvate.

These findings suggest that malic enzymes and the pyruvate-malate cycle can contribute to cycling pathways that control fuel-stimulated insulin secretion, but there is currently no indication that these reactions are directly responsible for the generation of second messenger coupling factors.

1.4.2 Pyruvate-Citrate Cycling

In addition to malate, other TCA cycle intermediates also increase during GSIS, including citrate and isocitrate (92). These metabolites are transported into the cytosol through the mitochondrial citrate/ isocitrate carrier (CIC) (108). Transport through the CIC has been shown to be a critical step for GSIS, as inhibition of the carrier using 1, 2, 3-benzene-tricarboxylate as well as siRNA-mediated suppression resulted in reduced insulin secretion (109). However, transport through the CIC is linked to several different metabolic pathways, including pyruvate-citrate cycling (Fig. 3) (109), fatty acid biosynthesis (110), and pyruvate-isocitrate cycling.

In pyruvate-citrate cycling, cytosolic citrate is first converted into oxaloacetate via citrate lyase (CL), then malate, and finally into pyruvate through the actions of MEc. Similar to MEc, inhibition of CL using hydroxycitrate as well as siRNA-mediated suppression failed to decrease GSIS (111), which indicates the pyruvate-citrate cycle is also not responsible for generating coupling factors necessary for insulin secretion.

Alternatively, conversion of citrate into oxaloacetate (and acetyl-CoA) is also the first step in the production of fatty acids; acetyl-CoA is then converted into malonyl-CoA by acetyl-CoA carboxylase (ACC1) and finally fatty acids by fatty acid synthetase (FAS), which can then in turn generate LC-CoA. Acute inhibition of ACC with 5-(tetradecyloxy)-2-furioc acid (TOFA) failed to decrease GSIS (112); furthermore, there is no insulin secretion phenotype in either the FAS-/- mouse (113) or in cells lacking FAS (111). As discussed previously in section 1.3.3, these results indicate the production of fatty acids does not play a role in regulating second phase GSIS and is not the critical second messenger signal generated by fuel metabolism.

1.4.3 Pyruvate-Isocitrate Cycling

Also downstream of CIC is the cytosolic NADP-dependent enzyme isocitrate dehydrogenase (ICDc), which catalyzes the conversion of isocitrate to α-ketoglutarate (2-oxoglutarate, 2OG) while reducing NADP+ to NADPH (Fig. 4). ICDc is somewhat unique in β-cells, which show minimal flux through the pentose-phosphate shunt, in that it is one of only a handful of enzymes capable of making cytosolic NADPH. Variations in production of NADPH have previously been positively correlated with GSIS, in a similar manner as flux through PC; furthermore, suppression of ICDc significantly decreased GSIS and NADPH production in two clonal β-cell lines (832/3 and 832/13), similar to the effects of CIC suppression, and also reduced GSIS in isolated primary rat islets (114). However, although these observations indicate an important role for the pyruvate-isocitrate cycle and ICDc in GSIS and suggest that NADPH may be a potential second messenger signal (114; 115), these experiments were not able to determine the relative importance of the other product of the ICDc reaction, 2OG. Moreover, the reactions through which the pyruvate-isocitrate cycle is completed in β-cells have not been identified. Indeed, recycling of TCA cycle intermediates to pyruvate may not be entirely necessary for GSIS, as 2OG itself has been implicated as a direct secretagogue (116).

2OG has several potential metabolic fates downstream of generation by ICDc, including direct conversion into other TCA cycle intermediates within the mitochondria, thereby completing a pyruvate-isocitrate cycle (without regenerating pyruvate). However, for this to occur, ICDc-derived 2OG must first be transported back across the inner mitochondrial membrane through the 2-oxoglutarate carrier.

1.4.4 The 2-Oxoglutarate Carrier

The 2-oxoglutarate carrier (OGC, SLC25A11) is part of a ~50-member family (in humans) of mitochondrial transporters, which includes the dicarboxylate carrier (DIC), ATP/ADP carrier (AAC), and uncoupling proteins (UCP) (117). These proteins all share a high sequence homology of three tandemly repeated ~100 amino acid peptide domains, and are responsible for the facilitated diffusion of solutes across the mitochondrial inner membrane. The carrier proteins are encoded by nuclear DNA, synthesized as non-cleavable mature-sized proteins, and enter the mitochondria through a different pathway than cleavable proteins (118-120). They play an important role in, among other processes, oxidative phosphorylation, fatty acid oxidation, transfer of reducing equivalents, and modulation of nucleotide pools in the mitochondrial matrix. A number of diseases have been linked to mutations in various carriers including neonatal myoclonic epilepsy (SLC25A22) and Amish microcephaly (SLC25A19) (108).

The OGC is found within the inner mitochondrial membrane and is an electroneutral carrier, along with the monocarboxylate, dicarboxylate, and tricarboxylate carriers. It has been observed to act functionally as a monomer, but rapidly cycle between monomeric and dimeric forms, with the homodimer highly favored at equilibrium (121-123). The OGC is a bi-directional anti-porter, capable of transporting both 2OG and malate simultaneously in opposite directions, either into or out of the mitochondrial matrix. One of the main physiologic roles of the OGC in yeast is thought to be supply of 2OG for cytoplasmic biosynthesis of glutamate (124).

Interestingly, as discussed in more detail below, all fuel secretagogues are capable of making 2OG (125); this has lead to the idea that in addition to direct metabolism, 2OG itself may play a signaling role in the β-cell. Therefore, the OGC was chosen for study because it sits at a critical junction in 2OG metabolism, with manipulations to the OGC either facilitating or preventing return of 2OG to the oxidizing environment of the mitochondria. Within that framework, our investigations focused on altering 2OG transport with the hope that it might give us a better understanding of the importance of 2OG metabolism in control of insulin secretion.

1.5 Non-Glucose Fuel Secretagogues

In addition to stimulation by glucose, insulin secretion from β-cells can be induced or augmented in response to a wide variety of other compounds and substances.

The amino acid arginine stimulates insulin secretion, but through non-metabolic mechanisms. Instead, it directly depolarizes the β-cell membrane (126), similar to high extracellular concentrations of potassium.

As mentioned previously, fatty acids increase secretion in the presence of stimulating concentrations of glucose, through signaling via the GPR40 (68) receptor as well as altering glucose metabolism and pyruvate cycling (98). Similarly, the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) can also augment insulin secretion in the presence of glucose. These hormones, which signal through G-protein coupled receptor-mediated cascades, are produced by the intestinal L-cells or K-cells, respectively, and are released during feeding (127); the GLP-1 signaling pathway in particular has been targeted for development of therapies that increase insulin secretion in type II diabetes, which include Exendin-4 (GLP-1 receptor agonist, based on the venom of the lizard *Heloderma suspectum* (128)) and inhibitors of DPP-4 (which cleaves Exendin-4 and GLP-1) (129).

Regarding metabolic fuel secretagogues, pyruvate is able to stimulate insulin secretion in clonal immortalized INS-1 cell lines (832/13 and others), but is not a secretagogue in isolated islets (130). However, the sole reason for this appears to be that primary rat β-cells do not express the pyruvate transporter on their plasma membrane (131). Thus, metabolically, pyruvate alone is perfectly capable of stimulating insulin release in the β-cell.

Methyl esthers of TCA cycle intermediates, which are able to diffuse across cell and organelle membranes, are also effective as metabolic secretogogues, to varying degrees in cell lines and primary tissue. Dimethyl-succinate stimulates insulin secretion in rat but not mouse islets, and is metabolized even more effectively in the presence of acetoacetate (which provides acetyl-CoA for the citrate synthase reaction in the TCA cycle) (132).

Dimethyl-2OG (dm-2OG) has mixed reports on its function as a secretagogue. Willenborg, et al. observed that 15mM dm-2OG failed to stimulate insulin release in the absence of glucose (133). However, our lab and others (116) have used this compound to investigate insulin secretion in both immortalized β-cell lines and islets, and find that it is perfectly capable of triggering insulin release, although, as discussed later in chapter 3, with varying effectiveness between the β-cell lines and islets.

Another non-glucose fuel secretagogue is the amino acid glutamine. Upon exposure, extracellular glutamine enters the cytosol of the β-cell and is converted to glutamate through the glutaminase reaction (also producing ammonia). Glutamate is then able to participate in a number of other reactions within the cytosol, or it can be transported into the mitochondria through the aspartate/ glutamate (Slc25A12, Slc25A13) or glutamate (Slc25A18, Slc25A22) carriers (134). However, at this point glutamine and glutamate by themselves are unable to stimulate insulin secretion above baseline levels; instead, the β-cell must also be simultaneously exposed to compounds that activate the mitochondrial enzyme glutamate dehydrogenase (Glud1), which include the amino acid leucine or the non-hydrolyzable leucine analogue BCH (2-amino-2-norbornane carboxylic acid). Activation of Glud1 allows conversion of glutamate to 2OG (and vice-versa), thereby opening the door to metabolism in the TCA cycle. It is unclear, however, what additional events are necessary for the full stimulatory effect of glutamine, and how these compare with glucose as a secretagogue.

Interestingly, although Glud1 is found in all living organisms, only animal Glud1 is regulated by a large and diverse range of metabolites and post-translational modifications, which include inhibition by ATP, GTP, palmitoyl CoA, NADH, and ribosylation by SirT4 (135), balanced with activation by ADP and leucine (136). Such tight regulation suggests a pivotal role for Glud1 in animal metabolism. As mentioned, activation of Glud1 is a requirement for glutamine-stimulated insulin secretion in β-cells; additionally, activating mutations in Glud1, which eliminate inhibition by GTP, have been found to be responsible for neonatal hyperinsulinemia and abnormal insulin secretion in the presence of low concentrations of amino acids alone (137). For these reasons, Glud1 is an attractive target for investigating the processes responsible for insulin secretion.

1.6 Project Goals

In designing my thesis project investigating insulin secretion from pancreatic β-cells, the goal has been to expand upon previous research addressing two main areas. The first is determining the contributions of various metabolic pathways in stimulating insulin release from β-cells. The second area is identification of the second-messenger coupling factor(s) responsible for second-phase ATP-independent insulin secretion.

To that end, my research initially investigated enzymes that were differentially expressed between high-responding and low-responding β-cell lines, and covered a diverse range of metabolic processes including branched-chain amino acid metabolism (Hibadh, Bdk), isomerase reactions in beta-oxidation of fatty acids (Ech), and synthesis of monounsaturated fatty acids (Scd). However, while we are continuing to investigate other aspects of β-cell metabolism, the bulk of my thesis project has focused on two genes closely linked to the pyruvate cycling reactions, OGC and Glud1, and their roles in insulin secretion.

My initial hypothesis for this project was that if the generation of either cytosolic or mitochondrial 2OG was the sole critical signal for insulin secretion, then OGC suppression would be presumed to leave either glucose- or glutamine-insulin secretion unaffected, as each fuel directly produces 2OG in either cytosolic or mitochondrial compartments, respectively. Likewise, if cytosolic production of 2OG was important, dm-2OG-stimulated insulin secretion should also remain unaffected by OGC suppression. However, if OGC suppression reduced insulin secretion by all forms of fuel stimulation, this would indicate the entire pyruvate-isocitrate cycling pathway as a whole needs to remain intact. Results of these studies are described in chapter 3.

As a complementary extension to our interest in the role that 2OG metabolism plays in insulin secretion, I also investigated the effects of Glud1 suppression on both GSIS and AASIS, in a manner similar to the OGC studies. Here, I hypothesized that any effects of Glud1 suppression on AASIS would be due to decreased glutamine oxidation, while a reduction in GSIS would instead be due to decreased production of second messenger coupling factors, indicating that Glud1 plays a different but no less important role in insulin secretion in response to glucose metabolism. Results of these studies are discussed in chapter 4.

Ultimately, the end goal of this combined research was not only to better understand the role of these specific components and their reaction pathways during insulin secretion in response to different fuel sources, but also to use these approaches to better pinpoint the potential second messenger signals produced by these pathways and correlate changes in these candidate coupling factors with alterations in insulin secretion.

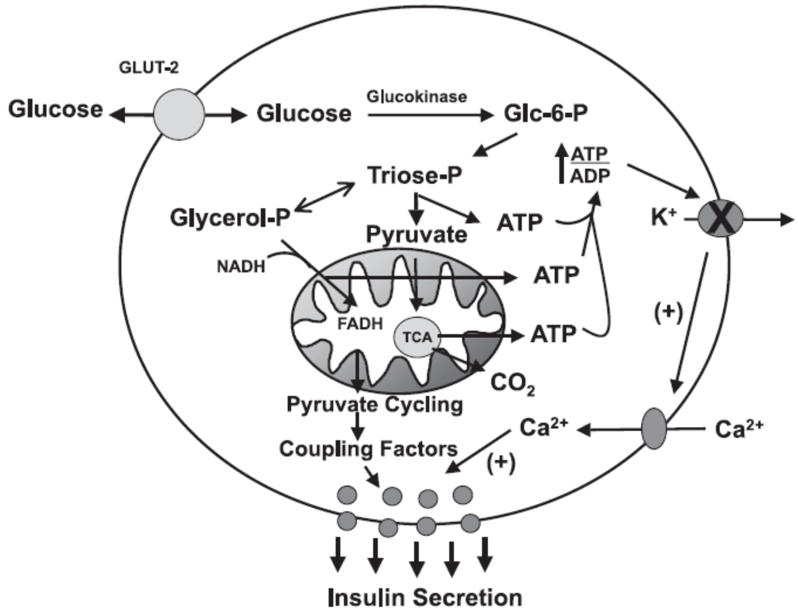

Figure 1: Mechanism of Glucose-Stimulated Insulin Secretion

Diagram showing the series of events necessary for GSIS, from Jensen, et al (23). Glucose is transported into the β-cell through the Glut-2 transporter, where it is then broken down through glycolysis in the cytosol, and oxidation in the TCA cycle within the mitochondria. Catabolism of glucose generates ATP, which increases the ratio of ATP:ADP and results in the closure of K_{ATP}-dependent ion channels followed by cell depolarization, which then leads to opening of L-type voltage-dependent calcium channels, influx of calcium, and ultimately activation of Ca^{2+}-dependent secretion machinery and insulin release. However, multiple lines of evidence indicate that addition ATP-independent coupling fators are also needed for the full secretion response, and are potentially generated through the pyruvate cycling reactions.

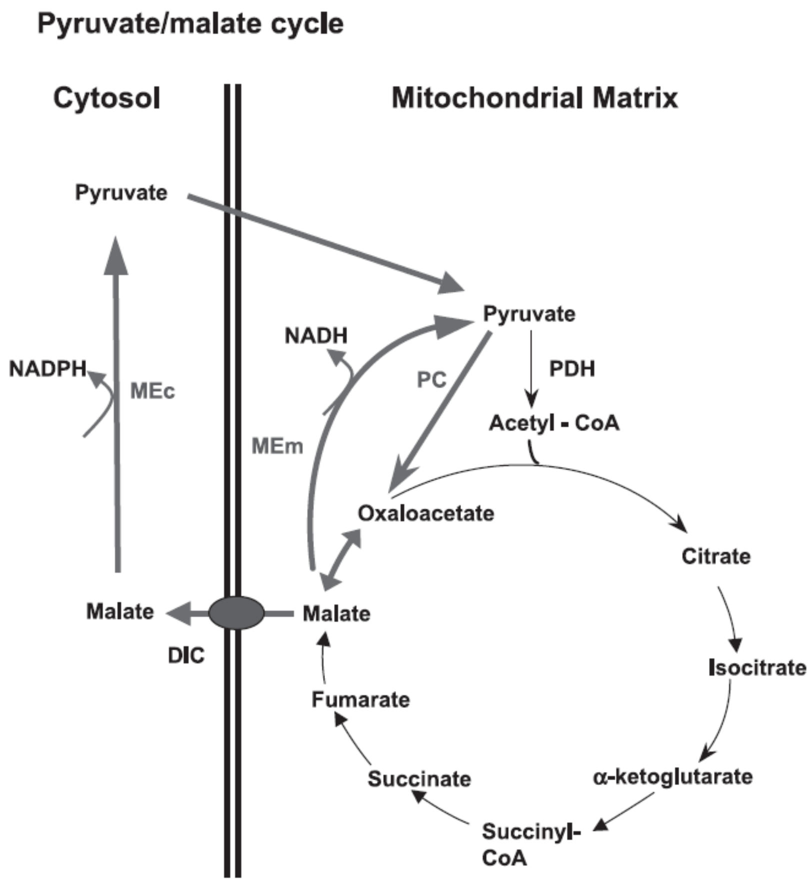

Figure 2: Pyruvate-Malate Cycling

Diagram showing the pyruvate-malate cycling reactions, taken from Jensen, et al (23). Mitochondrial malate can be directly converted into pyruvate through the actions of mitochondrial malic enzyme (MEm). Alternatively, malate can be transported into the cytosol through the dicarboxylate carrier (DIC), and reconverted into pyruvate by cytosolic malic enzyme (MEc).

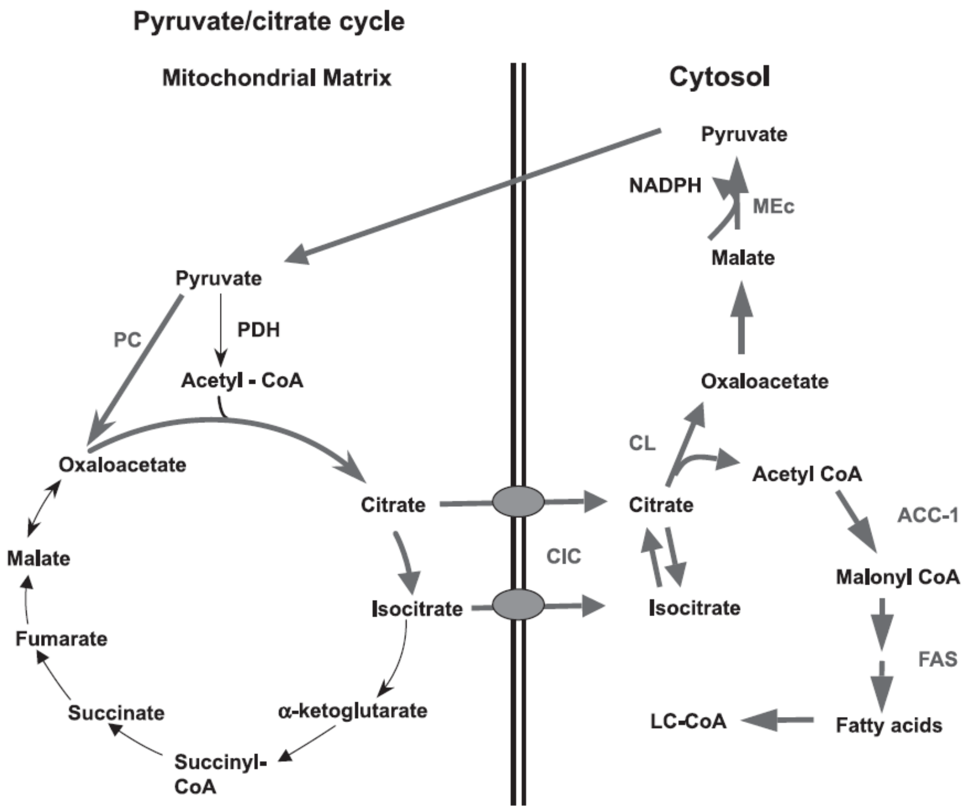

Figure 3: Pyruvate-Citrate Cycling

Diagram showing the pyruvate-citrate cycling reactions, taken from Jensen, et al (23). Citrate and isocitrate are transported into the cytosol through the citrate/ isocitrate-carrier (CIC). Citrate can then be converted back into pyruvate through the actions of citrate lyase (CL), malate dehydrogense, and malic enzyme (MEc), while the acetyl-CoA generated by the CL reaction can be used for the production of long chain-CoA (LC-CoA).

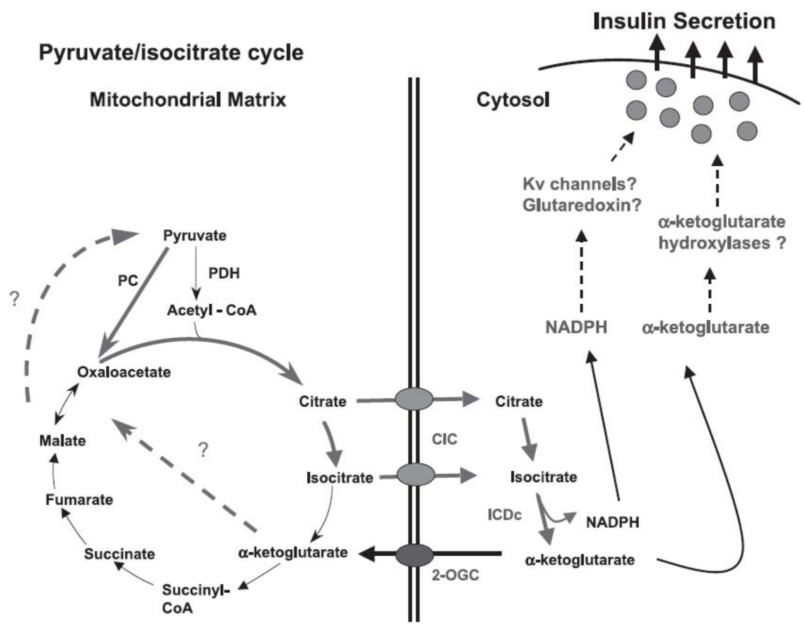

Figure 4: Pyruvate-Isocitrate Cycling

Diagram showing the pyruvate-isocitrate cycling reactions, taken from Jensen, et al (23). Cytosolic isocitrate is converted into α-ketoglutarate (2OG) through the actions of cytosolic NADP-dependent isocitrate dehydrogenase (ICDc). 2OG can then be transported back into the mitochondria through the OGC. Changes in ICDc expression and activity have previously been shown to alter NADPH production and insulin secretion; several potential mechanisms for these effects are shown.

2. Experimental Procedures

Reagents—All reagents were purchase from Sigma-Aldrich Chemical Company (St. Louis, MO), unless otherwise stated.

Adenovirus construction and purification—For overexpression of the OGC, the OGC gene was first amplified from rat 832/13 β-cell cDNA with the SuperScript™ III One-Step RT-PCR System with Platinum Taq® High Fidelity (invitrogen) using the forward primer AATC-GAATTC-CAAAGCCGAGGGCCATCAAG containing an EcoR1 restriction site and 4-base leader sequence, and reverse primer AATC-AAGCTT-TGGAAACCCTGGCACACGAG containing a HinDIII restriction site and 4-base leader sequence. The PCR product (1020 bases) was gel purified using the Qiaex II Gel Extraction Kit (Qiagen), digested overnight at 37˚C using EcoR1 and HinDIII enzymes (Roche Applied Science), and ligated into EcoR1/ HinDIII double-digested pAC.CMV plasmid, which was then used to construct the overexpression adenovirus, AdCMV-OGC, through co-transfection of HEK293 cells with plasmid JM17, as previously described (138-140). For overexpression studies of Glud1, the wild-type Glud1 gene was cloned by Danhong Lu (Newgard Lab, Duke University), and used to create the Glud1 overexpression adenovirus, AdCMV-Glud1. For suppression of the OGC, oligos containing the target sequence GCAATTCTTGCTGGACTCA or CTAGCATCCTGAAGGCAGA were annealed and ligated into the plasmid vector FF805 to generate adenoviruses Ad-siOGC#1 and Ad-siOGC#2, respectively, using the methods previously described. For suppression of Glud1, oligos containing the target sequence CTACAAGTGTGCAGTGGTT or GACGTTTGTTGTTCAGGGA were similarly used to generate siRNA viruses Ad-siGlud1-1 and AdsiGlud1-2, respectively. Adenoviruses were used to transduce HEK293 cells, which were scraped into 2ml of freeze-thaw buffer (10mM Tris pH = 8.0, 1mM $MgCl_2$) just prior to lysing (2 days after transfection). Samples were subjected to three freeze-thaw cycles, then purified by CsCl gradient (1hr spin at 191,000 x g over layered 1.2, 1.33, and 1.45g/ml CsCl solutions), and dialyzed in 10,000 molecular-weight cutoff cassettes (Pierce, Rockford, IL) in four 1-liter changes of freeze-thaw buffer at 4°C over two days. Virus titers were estimated by measuring OD and multiplying by 1 x 10^{12}; all viruses had titers between 2 x 10^{12} and 5 x 10^{13}. Aliquots were stored in 10% glycerol at -80°C, and diluted prior to use in experiments (~0.2ul of 5 x 10^{12} virus / well of a 12-well plate, for 832/13 cells).

Tissue culture—The 832/13 β-cell line (89), derived from INS-1 insulinoma β-cells (88), was used in these studies. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 10mM sodium HEPES, 2mM glutamine, 1mM sodium pyruvate, and 50μM β-mercaptoethanol, without antibiotics, at 37°C in 5% CO_2. For adenovirus transduction experiments, 832/13 cells were plated at a density of 5 x 10^6 cells/ 12-well plate. The following day, cells were treated with purified virus for 16 hours, followed by culture for 3 additional days with the medium changed daily prior to assays, as previously described (111).

Islet experiments—Pancreatic islets were isolated from Sprague-Dawley rats as previously described (141), with modifications (142). For adenovirus transduction experiments, immediately following isolation (day 0) islets were washed three times in RPMI incubation media (RPMI containing 8mM glucose, 10% fetal bovine serum, 20U/ml penicillin, 20ug/ml streptomycin, and 0.05ug/ml amphotericin B (Gibco, Carlsbad, CA), then incubated overnight for 16 hrs in 500ul RPMI culture media containing adenovirus. The following morning (day 1), the islets were transferred to 3ml of new RPMI media. Media was changed again each morning for the next two days (days 2 and 3), with insulin secretion assays being conducted on the fourth day after transduction (day 4).

Insulin secretion assays—Virus-treated 832/13 cells were preincubated for 2 hours in Krebs-Ringer bicarbonate (KRB) solution (4.38mM KCl, 1.2mM $MgSO_4$, 1.5mM KH_2PO_4, 129mM NaCl, 5mM $NaHCO_3$, 3.11mM $CaCl_2$, 10mM HEPES, and 0.25% BSA, pH = 7.4) containing 2mM glucose, then incubated for 2 hours in 2mM glucose, 12mM glucose, 12mM glutamine, 12mM glutamine + 6mM BCH, or 12mM dm-2OG. Afterwards, samples were collected for protein quantification and realtime PCR analysis, described below. For islets, insulin secretion was conducted by first incubating each islet treatment group together for 1 hr in 3-5ml of KRB plus 2mM glucose. The islets were incubated in groups of 20-30 in 500ul KRB buffer containing 2mM glucose, 12mM glucose (basal and stimulatory glucose), 12mM glutamine, 12mM glutamine + 12mM BCH (basal and stimulatory glutamine), or a range of concentrations for dimethyl-2-oxoglutarate (2-12mM). Afterwards, islets were then used for measurements of insulin content, protein, and gene expression by realtime PCR. Insulin release from both cells and islets was quantified by radioimmunoassay (RIA) Coat-a-Count kit (DPC, Los Angeles, CA) as described previously (89; 143).

Real time PCR—RNA was isolated from 832/13 cells using the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD) or from islets using the RNeasy Micro Kit, which included treatment of samples with DNase (Qiagen). Then 0.5-1ug sample RNA was reverse transcribed using the iScript cDNA synthesis kit (BioRad, Hercules CA). Real time PCR was performed for OGC expression using iTaq SYBR Green Supermix with ROX (Biorad), containing 100nM forward primer AAAGCCCTGATTGGCATGAC and reverse primer ATGGAAGCAGCAGTGGTGAC. Glud1 expression was measured similarly, using forward primer ACGACCCCAACTTCTTCAAG and reverse primer TCACCTCATCCACACTCACG. Cyclophilin B was measured as an internal loading control, using forward primer CGGACAGCCGGGACAA and reverse primer TTCGATCTTGCCACAGTCTACAA. Measurements and analyses were performed on an ABI Prism 7000 Sequence Detection System.

Protein and Insulin Content Measurements—For protein quantification, samples from 832/13 cells and islets were collected and lysed by several freeze-thaw cycles in Sigma M-cell lysis buffer. Then total sample protein was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Insulin content was determined by addition of 0.1M acetic acid plus 0.1% BSA to samples, followed by brief sonication and one freeze-thaw cycle. Samples were then diluted 1:50 in PBS, and insulin determined by RIA, as before.

Western Blotting—Rabbit antisera against the rat OGC peptide fragment IQNMRMIDGKPEYKN was generated by Antagene, Inc. (Mountain View, CA). For immunoblotting, mitochondria were first isolated from virus-treated 832/13 cells using the Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific, Inc.), and lysed in Cell Lytic™ M Cell Lysis Reagent (Sigma) using three freeze-thaw cycles. 50ug of protein per sample was separated on a 10% bis-tris gel, transferred to a PVDF membrane, blocked for 1hr at RT in TBS plus 2% BSA, 0.05% TWEEN® 20 (Sigma), and incubated overnight at 4°C in antisera diluted 1:1000 in TBS plus 0.05% TWEEN® 20 and 1% PVP. Blots were washed in TBS, incubated at RT for 1 hr in 2° anti-rabbit antibody (Sigma). Expression of Glud1 protein was also determined by western blotting of isolated mitochondria from control cells and cells transduced with adenovirus. 20ug of protein was loaded per sample, ran across a 4-12% Bis-Tris gel in MOPS buffer, run at 200V for 50 minutes, and transferred to a PVDF membrane at 30V for 1 hr. Membranes were similarly blocked, washed, and incubated overnight at 4°C with rabbit anti-bovine Glud1 Antibody (Cat. #G4000-50, US Biological, Swampscott, MA), diluted 1:1,000 in TBS containing 0.05% Tween and 1% polyvinylpyrrolidine (PVP). After incubation, membranes were again washed twice in TBS at RT for 10 minutes, and incubated for 1hr at RT in anti-rabbit HRP (GE Healthcare) diluted 1:10,000 in TBS containing 0.05% Tween and 1% PVP. All blots were developed using the ECL detection system (GE Healthcare) and visualized on the VersaDoc 4000 Imaging System (Bio-Rad). Results were normalized to the expression of VDAC-1 (mouse monoclonal ab14734, abcam, Cambridge, MA), which was used as a mitochondrial protein loading control.

2-Oxoglutarate Carrier Transporter Assay—Virus-treated 832/13 cells were washed with PBS, scraped into 1.5ml eppendorf tubes, and washed twice more with cold PBS + 0.02% EDTA. Supernatant was removed, and 650ul permeabilization buffer (100mM KCl, 22mM NaCl, 10mM K*HEPES, 1mM $MgCl_2$, 5mM $KHCO_3$) plus 13ul Saponin Solution (40mg hemolytic reagent Saponin (Sigma), 5.0ml H_2O) was added to each tube to lyse the plasma membrane of the cells while leaving the mitochondria intact. Samples were left at RT for 25 minutes, then cell pellets were washed twice in permeabilization buffer without Saponin, and incubated at 37˚C in permeabilization buffer plus 12mM glutamic acid, 1mM malic acid, and 6mM BCH. Activated glutamate dehydrogenase in the intact mitochondria converted glutamate to 2-oxoglutarate, which was then transported out of the mitochondria at a rate directly dependent on the amount of OGC present in the mitochondrial membrane. After 45 minutes, supernatant containing newly-generated 2-oxoglutarate was removed and combined with ICD reaction buffer (40mM $MgCl_2$, 35mM $NaHCO_3$, 100mM Na-HEPES, 10% glycerol, 80uM NADPH, and .25U/ reaction NADP-dependent Isocitric Dehydrogenase enzyme (Sigma)), based on the

ICD reaction used by Kanao, et al (144). Relative OGC transporter activity for each treatment was then determined by quantifying the rate of NADPH consumption by the ICD-catalyzed reaction (2-oxoglutarate to isocitrate) through kinetic measurements of the change in absorbance at 340nm. It is important to note that this *in vitro* assay forced the ICD reaction to run in the opposite direction from what is expected to occur in the intact β-cell.

Glud1 Activity Assay—The *in vitro* activity of Glud1 was measured using the following procedure adapted from Fujioka, et al (145). Mitochondria were first isolated from cells by dounce homogenization followed by centrifugation over a sucrose gradient (114), and lysed in homogenization buffer (10mM Tris-acetate (pH = 8.0), 1mM EDTA, 0.5% Triton X-100, 3.95ml H2O, pH to 8.0). Then, the rate of NADH consumption was measured in reaction buffer (20mM Tris-Acetate (pH = 8.0), 50mM NH_4Cl, 0.2mM NADH, 1mM EDTA, 0.5% Triton X-100, 6mM BCH, and 1.4ml H_2O), by recording the change in absorbance at 340nm after addition of 10mM 2-oxoglutarate.

Glucose usage—Glucose usage (glycolytic flux) in virus-treated 832/13 cells was determined by incubation for 2 hrs in 0.75ml/well (12-well plate) of 2mM or 12mM glucose with 0.06 Ci/mol 5-^{3}H-glucose (GE Healthcare) added as tracer, as described previously (146). At the end of the incubation period, 500ul incubation media was collected and protein precipitated by addition of 200ul 10% TCA. Samples were centrifuged for 2 min at 12,000 x g, and 200ul of supernatant was transferred to a capless eppendorf tube. The tube was then place in a scintillation vial containing 500ul H_2O, and the system was capped and allowed to equilibrate overnight at 50°C. The following day, the eppendorf tubes were removed from the scintillation vials, and 5ml of scintillation fluid was added. The samples were then counted by liquid scintillation. Efficiency was determined by measuring the equilibration of ^3H_2O.

Glucose Oxidation—Glucose oxidation was measured in virus-treated 832/13 cells using a CO_2 trapping system (147) , as previously described (109; 146). Cells were incubated in 0.5ml/ well (12-well plate) of 2mM or 12mM glucose containing 0.5 Ci/mol U-^{14}C-D-glucose as tracer. At the end of the incubation period, 300ul of buffer was removed and transferred to a capless 500ul eppendorf tube, placed inside a scintillation vial containing 200ul 1M NaOH. A rubber stopped was used to cover the scintillation vial, and a syringe used to inject 100ul of 70% perchloric acid into the eppendorf tube, acidifying the sample. Samples were left at room temperature for 1 hr, then the rubber stopper and eppendorf tubes were removed. 5ml of Uniscint BD scintillation fluid (National Diagnostics, Atlanta, GA) was added to each tube, and samples were counted by liquid scintillation.

Glutamine Oxidation—Oxidation of glutamine was measured similar to glucose, by incubation of cells for 2 hrs in KRB secretion buffer containing 12mM glutamine plus 6mM BCH with [U-^{14}C] glutamine as a tracer (0.1 Ci/ Mol). Following the 2hr incubation, the buffer was removed and transferred to 600ul cup within a CO_2 trapping system, containing 200ul 1M NaOH at the bottom of a scintillation vial, with one vial per sample. The system was covered with a rubber stopper, the media was acidified with addition of 70% perchloric acid, and the vials placed on a shaker at RT. After 1 hr, the stoppers were removed along with the cup, and 5ml of high-salt compatible scintillation fluid was added to each vial. Glutamine oxidation was then determined using liquid scintillation counting of ^{14}C-CO_2 produced.

MTS assay—Cell viability and general mitochondrial function were determined using the CellTiter 96® Proliferation Assay (Promega, Madison, WI). 20ul reagent was added to 1ml of KRB buffer during the last hour of incubation phase of the insulin secretion assay. Then 200ul of each sample was used for measurement of absorbance at 490nm, with color change indicating reduction of the MTS tetrazolium compound.

ATP and ADP—Measurements of ATP and ADP were determined using a Luciferase ATP assay (Sigma), as previously described (143; 148). Virus-treated 832/13 cells on 6-well plates were stimulated with either low or high glucose, or glutamine or glutamine plus BCH. At the end of the 2hr incubation period, cells were washed in ice cold PBS and scraped into 1.5ml eppendorf tubes. Samples were briefly spun at ~1,000 x g to pellet the cells, the supernatant was removed, and the cell pellet snap-frozen in a dry-ice ethanol bath. Samples were then stored at -80°C prior to the assay. The cell pellet samples were then extracted with 100ul 0.3N PCA and left on ice for 30 minutes. Samples were neutralized with 2N KOH/ 0.5M triethanolamine, and again left on ice for

10 minutes to precipitate PCA prior to being centrifuged at 12,000 x g for 5 minutes at 4°C. Then 10ul for taken to measure ATP, and 70ul of sample was used to measure ADP. For ATP measurements, ATP assay mix (Sigma) was dissolved in water then combined with assay buffer (Sigma) at a 1:3 ratio, and used at 100ul/ reaction plus 10ul ATP sample. Luminescence at 592nm was read several times over the course of 10 minutes. For ADP measurements, the 70ul ADP sample was combined with 50mM Tris (pH = 8.0), 5mM $MgCl_2$, 10mM $NaMoO_4$, 10mM GMP, and 0.25U AMP-sulfurylase (plus water up to 200ul total), which converts ATP to AMP. The reaction was run for 50 minutes at 30°C, followed by 10 minutes at 95°C to inactivate the enzyme. After a 5 minute centrifugation at 12,000 x g, 100ul was used for a second reaction with 47mM Tris (pH = 8.0), 47mM $MgCl_2$, 38mM KCl, 0.5mM phosphoenol pyruvate, and 8U pyruvate kinase (plus water to 200ul total), which converts the remaining ADP to ATP. This reaction was run at 26°C for 30 minutes, followed by 95°C for 5 minutes. Then 100ul of the reaction was combined with 33ul ATP assay mix and 66ul ATP assay buffer, and luminescence was measured as before.

NADPH assay—NADP+ and NADPH were measured in virus-treated 832/13, as previously described (149) with modifications (92; 115). After GSIS or AASIS, cells from 6-well plates were washed with cold PBS, scraped into 1.5ml eppendorf tubes (on ice), centrifuged, and cell pellets stored at -80°C. Cell pellets were then thawed on ice and resuspended in 50ul 40mM NaOH plus 5mM cysteine. Samples were then briefly sonicated and centrifuged for 1 minute at 10,000 x g at 4°C. Three 10ul aliquots of each sample with 0.3M HCl, 40mM NaOH, or 5mM cysteine added to each aliquot for measurements of NADP+, NADPH, or total NADP(H), respectively, as NADPH is destroyed by low pH and NADP+ is destroyed by high pH. Samples for NADPH and NADP+ determinations were incubated at 60°C for 15 min, while the sample for NADP(H) determination was kept on ice. Standards were treated in parallel. Cycling reagent comprised of 50mM imidazole, 50mM imidazole-HCl, 5mM glucose-6-phosphate, 7.5mM disodium 2-oxoglutarate, 0.1mM ADP, 25mM ammonium acetate, 0.1% BSA, 1.4U/ml *Leuconostoc* glucose-6-phosphate dehydrogenase, and 1.5U/ml beef liver glutamate dehydrogenase (pH = 7.0) were added, and samples were incubated for 3 hrs at 37°C. The reaction was stopped by boiling samples for 3 min, followed by centrifugation at 10,000 x g for 10 min. The amount of 6-phosphogluconolactone generated was determined through use of an indicator reaction; 50mM imidazole, 50mM imidazole-HCl, 30mM ammonium acetate, 2mM $MgCl_2$, 0.1mM EDTA, 300μM NADP+, and 0.5U/ml *Torula* yeast gluconate dehydrogenase was added to the cycling reactions, and absorbance was measured at 340nM following kinetic reaction completion.

Measurements of Amino Acids—Amino acids were analyzed from virus-treated cells after GSIS or AASIS by tandem mass spectrometry (MS/MS) with a Quattro Micro instrument (Waters Corporation, Milford, MA)(150; 151). The acidic conditions used to form butyl esters results in partial hydrolysis of glutamine to glutamic acid and asparagine to aspartate. Accordingly, values reported as Glu/Gln or Asp/Asn measure the amount of glutamate or aspartate plus the contribution of the partial hydrolysis reactions of glutamine and asparagine, respectively.

Statistical methods—Statistical differences were determined using Student's t-test ($p < 0.05$). Data are presented as mean ± SEM, for $n \geq 3$ separate experiments.

Table 1: DNA oligo sequences

Oligo name	Gene	Use	Sequence
SYBR, rSlc25a11, F	OGC	realtime	AAAGCCCTGATTGGCATGAC
SYBR, rSlc25a11, R	OGC	realtime	ATGGAAGCAGCAGTGGTGAC
KGC#1 Forward + EcoR1	OGC	cloning	AATCGAATTCAAAAGCCGAGGGCCATCAAG
KGC#1 Reverse+HinDIII	OGC	cloning	AATCAAGCTTTGGAAACCCTGGCACACGAG
siKGC 192105 sense	OGC	siRNA	GATCCCCGCAATTCTTGCTGGACTCATTCAAGAGA-TGAGTCCAGCAAGAATTGCTTTTTGGAAA
siKGC#1 antisense, 05	OGC	siRNA	AGCTTTTCCAAAAAGCAATTCTTGCTGGACTCATCT-CTTGAATGAGTCCAGCAAGAATTGCGGG
Forward siOGC-IDT1	OGC	siRNA	GATCCCCCTAGCATCCTGAAGGCAGATTCAAGAGA-TCTGCCTTCAGGATGCTAGTTTTTGGAAA
Reverse siOGC-IDT1	OGC	siRNA	AGCTTTTCCAAAAACTAGCATCCTGAAGGCAGATCT-CTTGAATCTGCCTTCAGGATGCTAGGGG
SYBR-Glud1 F	Glud1	realtime	ACGACCCCAACTTCTTCAAG
SYBR-Glud1 R	Glud1	realtime	TCACCTCATCCACACTCACG
siGlud1-IDT1, F	Glud1	siRNA	GATCCCCCTACAAGTGTGCAGTGGTTTTCAAGAGA-AACCACTGCACACTTGTAGTTTTTGGAAA
siGlud1-IDT1, R	Glud1	siRNA	AGCTTTTCCAAAAACTACAAGTGTGCAGTGGTTTCT-CTTGAAAACCACTGCACACTTGTAGGGG
siGlud1-IDT2, F	Glud1	siRNA	GATCCCCGACGTTTGTTGTTCAGGGATTCAAGAGA-TCCCTGAACAACAAACGTCTTTTTGGAAA
siGlud1-IDT2, R	Glud1	siRNA	AGCTTTTCCAAAAAGACGTTTGTTGTTCAGGGATCT-CTTGAATCCCTGAACAACAAACGTCGGG
Cyclo F, SYBR	Cyclophilin	realtime	CGGACAGCCGGGACAA
Cyclo R, SYBR	Cyclophilin	realtime	TTCGATCTTGCCACAGTCTACAA

Table 2: siRNA target sequences

Sequence name	Gene of Interest	Sequence Target
siOGC1	2-oxoglutarate carrier	GCAATTCTTGCTGGACTCA
siOGC2	2-oxoglutarate carrier	CTAGCATCCTGAAGGCAGA
siGlud1-1	glutamate dehydrogenase	CTACAAGTGTGCAGTGGTT
siGlud1-2	glutamate dehydrogenase	GACGTTTGTTGTTCAGGGA

3. Flux through the Mitochondrial 2-Oxoglutarate Carrier is Required for Fuel-Stimulated Insulin Secretion

Glucose-stimulated insulin secretion (GSIS) from pancreatic islet β-cells is dependent in part on pyruvate cycling through the pyruvate-isocitrate pathway, which generates cytosolic α-ketoglutarate, also know as 2-oxoglutarate (2OG). To further understand the role of 2OG metabolism in insulin secretion, we investigated mitochondrial transport of 2-oxoglutarate through the 2-oxoglutarate carrier (OGC). Suppression of OGC expression in clonal pancreatic β-cells (832/13 cells) and in isolated rat islets using adenovirus-mediated siRNA transduction significantly decreased GSIS. Additionally, a greater reduction in GSIS was seen in islets after combined suppression of the OGC and the dicarboxylate carrier (DIC). OGC suppression also reduced insulin secretion in response to glutamine plus the glutamate dehydrogenase (GDH) activator BCH. Glucose usage, glucose oxidation, glutamine oxidation or the increase in the ATP:ADP ratio were not affected by OGC knockdown. Suppression of OGC expression did result in a significant decrease in the NADPH:NADP+ ratio after stimulation with glucose, but not after stimulation with glutamine plus BCH. Finally, OGC suppression also reduced insulin secretion in response to stimulation with dimethyl-2OG. These data therefore support the hypothesis that the OGC is part of common mechanism of fuel-stimulated insulin secretion, but indicate that NADPH production per se may not be a universal requirement for all β-cell secretagogues.

3.1 Introduction

Insulin secretion from pancreatic β-cells occurs in response to catabolism of metabolic fuels, and is necessary for maintenance of plasma glucose homeostasis. Glucose-induced increases in the cytosolic ATP:ADP ratio play an important role in triggering insulin release by closing ATP-dependent potassium (K_{ATP}) channels (49), leading to a cascade of events involving cell depolarization, influx of calcium, and insulin vesicle binding with the plasma membrane. However, multiple lines of evidence have emerged in recent years that implicate additional signals derived from fuel catabolism in control of insulin secretion, which are independent of changes in ATP:ADP ratio (52; 152). As dysregulation of insulin secretion marks the transition from pre-diabetes to overt hyperglycemia and type 2 diabetes, better understanding of these ATP-independent signals is necessary to identify potential pharmacologic targets and facilitate development of new drug therapies.

In β-cells, anaplerotic entry of pyruvate into the TCA cycle through PC has been strongly correlated with glucose-stimulated insulin secretion (GSIS) (90), and leads to mitochondrial efflux of citrate, isocitrate, and malate into the cytosol (30). Once in the cytosol, these metabolic intermediates can be reconverted into pyruvate through several reaction pathways, termed "pyruvate cycling reactions". Extensive investigation by our lab has revealed that the pyruvate-malate and pyruvate-citrate cycles do not play a major role in regulating insulin release (23; 105). Instead, suppression of cytosolic NADP-dependent isocitrate dehydrogenase (ICDc), a key enzyme in the pyruvate-isocitrate cycle, significantly reduces insulin secretion (114), which indicates a key role for this reaction pathway in regulating insulin release. The products of the ICDc reaction are cytosolic NADPH, and α-ketoglutarate, also know as 2-oxoglutarate (2OG).

Both NADPH and 2OG have been suggested as potential ATP-independent coupling factors for insulin secretion. Interestingly, the most potent fuel secretogogues are all capable of generating 2OG (125), indicating its potential importance in stimulus-secretion coupling. Stimulation of β-cells with a non-glucose fuel secretagogue, glutamine plus leucine (leucine is an activator of glutamate dehydrogenase), results in direct conversion of glutamate to 2OG within the mitochondria and robust amino acid-stimulated insulin secretion (AASIS) (153). Even so, the contribution of 2OG metabolism to insulin secretion, either via direct influence as a coupling factor or maintenance of flux through various pyruvate cycling pathways, remains to be determined. Furthermore, the role of NADPH in regulating insulin secretion in response to non-glucose fuels is also unknown.

Therefore, in the current study we used adenovirus transduction of 832/13 clonal β-cells and isolated rat islets to either overexpress or suppress expression of the 2-oxoglutarate carrier (OGC, SLC25A11) (154; 155), and tested the hypothesis that 2OG transport through the OGC is necessary for robust GSIS and/ or AASIS. Furthermore, we also investigated the effects of carrier suppression on cell metabolism, including NADPH production after stimulation with glucose or glutamine. Our results indicate that while the OGC is part of a common pathway of reactions necessary for insulin secretion in response to multiple fuel substrates (including glucose, glutamine, and dimethyl-2-oxoglutarate), production of NADPH may not be a universal signal for regulating insulin release.

3.2 Results

Overexpression of the OGC in 832/13 β-cells and isolated rat islets fails to alter GSIS

To investigate the role of 2OG transport in insulin secretion from β-cells, recombinant adenovirus was used to overexpress the rat 2-oxoglutarate carrier (OGC). Overexpression using virus AdCMV-OGC resulted in a 10-fold increase (p<0.05) in OGC mRNA expression in 832/13 β-cells as compared to treatment with a virus overexpressing β-galactosidase, AdCMV-β-Gal (Fig. 5a). OGC protein was also increased by 410 ± 54% (p<0.01) as observed by immunoblotting of mitochondrial extracts (Fig. 5b). As a further demonstration of OGC overexpression, transport of 2OG through the mitochondria was assayed using a linked reaction in cells permeabilized with the hemolytic reagent Saponin, as described in the methods section. Overexpression of the OGC resulted in an almost 5-fold increase (p<0.05) in 2OG transport (Fig. 5c). Despite this clear increase in OGC expression and activity in

AdCMV-OGC-treated cells, no effect on glucose-stimulated insulin secretion from 832/13 β-cells was observed, as compared with AdCMV-βGAL-treated cells (*p>0.7)* (Fig. 6). Similarly, while overexpression of the OGC in isolated rat islets greatly increased OGC mRNA ($p<0.05$) (Fig. 7a), no effects were observed on GSIS (Fig. 7b)

Suppression of the OGC in 832/13 β-cells reduces GSIS

Treatment of 832/13 β-cells with viruses Ad-siOGC#1 and Ad-siOGC#2 resulted in a dose-dependent decrease in OGC mRNA levels, with maximal effects of 83 ± 6% ($p<0.001$) and 71 ± 10% ($p<0.01$) suppression at the highest viral doses used, respectively (Fig. 8b, Fig. 9b). Suppression of the OGC using the most effective virus, Ad-siOGC#1, resulted in a 65 ± 10% decrease ($p<0.01$) in the rate of 2OG transport through the mitochondria (Fig. 10a), and a corresponding 52.7 ± 4.3% decrease ($p<0.001$) in OGC protein expression as determined by immunoblot analysis (Fig. 8c). These viruses also caused a dose-dependent reduction in glucose-stimulated insulin secretion. At the highest dose, Ad-siOGC#1 decreased GSIS by 56.8 ± 10.7% ($p<0.05$), while Ad-siOGC#2 decreased GSIS by 48.0 ± 14.3% ($p<0.5$), as compared to treatment with a scrambled control siRNA virus, Ad-siControl (Fig. 8a, Fig. 9a). Suppression of the OGC also resulted in a 42.6 ± 9.5% ($p<0.05$) reduction in insulin secretion in response to high-glucose plus high-KCl, while no difference was observed under low-glucose plus high-KCl conditions (Fig. 10b).

Metabolic Assays of 832/13 β-cells after suppression of OGC

To determine if the effects of OGC suppression on GSIS were due to general alterations in metabolism, we next measured glucose usage, glucose oxidation, cell viability, and changes in the ratio of ATP:ADP before and after stimulation with glucose. After suppression of the OGC using siOGC#1 virus or siOGC#2 virus, no differences were observed in glucose usage (Fig. 11b), glucose oxidation (Fig. 11a), or cell viability as determined by MTS assay (Fig. 11c). A robust and similar increase in ATP:ADP ratio was observed in response to glucose stimulation in both Ad-siOGC#1- and Ad-siControl-treated cells (Fig. 11d).

NADPH production after stimulation with glucose is reduced by OGC suppression

Although no differences were observed in general metabolic function after suppression of the OGC, we reasoned that reduced 2OG transport could alter flux through the pyruvate-isocitrate cycling pathway. Suppression of two components of this pathway, cytosolic isocitrate dehydrogenase (ICDc) and the citrate/ isocitrate carrier (CIC), has previously been shown to decrease the production of NADPH in concert with suppression of GSIS (109; 114). Here, suppression of the OGC resulted in a 23.0 ± 7.0% ($P<0.05$) reduction in the ratio of NADPH:NADP+ after stimulation with glucose (Fig.

12). These results are consistent with a role for impaired NADPH production in mediating the effects of OGC suppression on GSIS.

Suppression of either ICDc or the OGC reduces glutamine-stimulated insulin secretion

To determine if other fuel secretagogues besides glucose share the same mechanism of stimulating insulin release, and to more thoroughly investigate the role of 2OG metabolism and transport in insulin secretion, glutamine-stimulated insulin secretion (AASIS) was investigated in 832/13 β-cells.

As a starting point, the effects of ICDc suppression on AASIS were studied, in part because the effects on GSIS had already been extensively characterized (114). Incubation of 832/13 cells with glutamine plus BCH resulted in robust insulin secretion comparable to glucose, while suppression of ICDc dramatically reduced both GSIS and AASIS to equal extents (Fig. 12b). These results suggest that glucose and glutamine are coupled to insulin secretion via a common metabolic pathway that includes cytosolic isocitrate dehydrogenase (ICDc).

Likewise, suppression of the OGC using Ad-siOGC#1 resulted in a 59 ± 14% ($P<0.05$) decrease in AASIS (Fig. 14a). This effect was achieved absent any impairment in the increase in the ATP:ADP ratio seen under stimulatory conditions (Fig. 14c), providing evidence of a K_{ATP} channel-independent component to AASIS. Furthermore, OGC knockdown caused no decrease in cell viability after stimulation with AASIS (Fig. 14b) or in glutamine oxidation (Fig. 14d).

NADPH production was also measured during AASIS. In comparison to glucose, stimulation of β-cells with glutamine plus BCH did not substantially increase NADPH production, and failed to produce much more than a doubling in the NADPH:NADP+ ratio (Fig. 15a). Therefore, our ability to detect changes in NADPH after suppression of either the OGC or ICDc was greatly hindered. While no differences were observed in NADPH production during AASIS after OGC suppression, it is surprising that suppression of ICDc, which directly generates NADPH, also failed to produce measurable changes in the NADPH:NADP+ ratio despite the fact that AASIS was reduced equivalently with glucose (Fig. 15b).

Insulin Secretion in islets after suppression of OGC

To investigate whether the effects on GSIS and AASIS translated to primary cells, insulin secretion from isolated rat islets was measured after suppression of the OGC. Treatment with Ad-siOGC resulted in a 71 ± 5% (p<0.001) decrease in OGC RNA as compared to Ad-siControl-treated islets (Fig. 16a); under these conditions, GSIS was reduced by 20 ± 5% (p<0.01) (Fig. 16b) and AASIS reduced by 23 ± 7% (p<0.05) (Fig. 16c).

Insulin Secretion after suppression of both the OGC and the DIC

Because the effect on GSIS and AASIS was not nearly as substantial in islets as in 832/13 cells, we hypothesized that primary cells may more metabolically flexible, and when necessary can place a greater emphasis on other mitochondrial transporters to complete pyruvate cycling, such as the dicarboxylate carrier (DIC). One possible route, shown in Fig. 19, is through the cytosolic conversion of 2OG first to OAA, and then to malate, which can enter the mitochondria through the DIC.

Therefore, both the DIC and the OGC were independently and/ or simultaneously suppressed in 832/13 cells and isolated primary rat islets, and GSIS was measured. Suppression of the DIC alone in 832/13 cells failed to alter GSIS, and had no additive effect on the reduction in GSIS when simultaneously suppressed along with the OGC (Fig. 17c). In contrast, suppression of the DIC in islets reduced GSIS to a similar extent at suppression of the OGC (Fig. 18c). Most interestingly, the combined suppression of the DIC and the OGC in islets resulted in an additive decrease in GSIS (Fig. 18d), indicating that in islets these two carriers may provide independent mechanisms for organic acid transport across the mitochondrial membrane (Fig. 19), thereby affecting metabolic pathways necessary for insulin secretion.

2OG-stimulated insulin secretion

To investigate the possibility that 2OG functioned as an extracellular autocrine or paracrine signaling molecule, insulin secretion was measured in response to dimethyl-

2OG (dm-2OG), which is membrane permeable, as well as the sodium salt of 2OG (ss-2OG), which is a charged molecule in solution and therefore unable to cross the plasma membrane. In cells, increasing concentrations of dm-2OG resulted in increased insulin secretion (Fig. 21a); however, the sodium salt form of 2OG failed to alter insulin release, regardless of the presence or absence of glucose (Fig. 20).

In contrast to the cell lines, increasing concentrations of dm-2OG in islets stimulated insulin release up to a concentration of ~2mM; however, beyond this concentration stimulation of insulin secretion was less and less pronounced, such that 12mM dm-2OG failed to increase insulin release substantially above basal levels (Fig. 21b). Islet perifusion experiments revealed that stimulation with 12mM dm-2OG did not stimulate second-phase insulin secretion, in contrast to 12mM glucose, which stimulated both first- and second-phase insulin release (Fig. 21c).

Based on these data, it was my hypothesis that direct stimulation of islets with supra-physiological levels of dm-2OG resulted in the rapid decrease in some factor responsible for maintaining appropriate metabolic flux rates and insulin secretion. One such possible factor was the amino acid aspartate, which can combine with 2OG in cytosolic or mitochondrial aspartate aminotransferase reactions (GOT1 or GOT2, respectively) and has been observed to decrease in β-cells during GSIS (156), suggesting that it may be a rate-limiting step in an aspect of metabolism necessary for insulin release.

Therefore, I used two different approaches to test the hypothesis that pre-loading β-cells with aspartate would increase their responsiveness to dm-2OG. First, islets stimulated with dm-2OG were also given an equimolar amount of asparagine, with the idea being that aspartate (made from asparagine) would then be available in ample supply for the GOT1 or GOT2 reactions. However, although insulin secretion was increased slightly by the addition of asparagine, the effect was small (Fig. 22b). The other approach focused on the 832/13 cell line, which are normally cultured in 2mM glutamine. Here, glutamine is likely normally taken up by the cells and converted into glutamate through the actions of the glutaminase enzyme. In this context, it is possible that these relatively high persistent levels of glutamate then force the GOT1 and GOT2 reactions to run in reverse, increasing the intracellular levels of aspartate and essentially "pre-loading" the cell line with aspartate prior to glucose stimulation. Interestingly, 24 hr culture in the absence of glutamate did lead to decreased dm-2OG-stimulated insulin secretion (Fig. 22a), although AASIS was not decreased. Additional investigation is therefore necessary to conclusively determine that reduced aspartate content was responsible for the effect on dm-2OG-SIS.

Because dm-2OG was such an effective secretagogue in the cell lines, I also measured 2OG-stimulated insulin secretion after OGC suppression. As with GSIS and AASIS, suppression of the OGC also decreased insulin secretion in response to 2OG (Fig. 23).

3.3 Discussion

In this study, we investigated the role of 2OG transport in fuel-stimulated insulin secretion from pancreatic β-cells, using adenovirus transduction to transiently overexpress or suppress expression of the mitochondrial 2-oxoglutarate carrier (OGC). Overexpression of the OGC failed to alter GSIS from either clonal β-cells or isolated rat islets, indicating that flux through the carrier under normal conditions is likely not a rate-limiting step in metabolism.

In contrast, suppression of the OGC using two unique siRNA targeting sequences resulted in significantly reduced GSIS. However, no changes were observed in glucose usage, glucose oxidation, cell viability or ATP production. The lack of change in glucose oxidation and general mitochondrial function after OGC suppression suggests a metabolic separation of TCA cycle flux from pyruvate cycling. This concept is supported by the previous observation that two separate pools of pyruvate exist in the β-cell (90), one that undergoes pyruvate cycling and one that is simply oxidized to meet energy needs. As β-cells have a limited capacity to increase glycolytic flux by increasing glycogen or lactate production (106), a potential role for the pyruvate-cycling reactions may be to shunt excess carbon away from oxidation. Within this paradigm, the changes in pyruvate cycling that occur during lipid overload (98; 99) may be necessary to prevent immediate detrimental effects on oxidative metabolism, even though glucose sensing and insulin secretion then become inpaired.

Suppression of the OGC also failed to decrease insulin release under conditions of low glucose plus a depolarizing concentration of KCl, which indicates that non-metabolic insulin secretion processes were unaffected. Instead, OGC suppression reduced insulin release in the presence of KCL plus high glucose, revealing that the carrier likely plays a critical role in the metabolic regulation of GSIS. With this result in mind, we proposed two potential explanations. The first was that decreased OGC transporter activity reduced either cytosolic or mitochondrial 2OG levels, thereby abrogating any signaling role the molecule might be playing in GSIS. An alternative hypothesis was that the entire pyruvate-isocitrate cycling pathway as a whole needed to remain intact for GSIS, with the requirement that 2OG transport through the OGC needed to remain unhindered.

To address these possibilities, both glutamine-stimulated insulin secretion (AASIS) and dimethyl-2OG-stimulated insulin secretion were measured after OGC suppression. AASIS directly generates mitochondrial 2OG via anaplerotic flux of glutamate through glutamate dehydrogenase; in contrast, stimulation of cells with dimethyl-2OG generates cytosolic 2OG. Suppression of the OGC reduced insulin secretion under both conditions, ultimately indicating that production of 2OG in any single compartment was unable to rescue insulin release; instead, in β-cells the pyruvate-isocitrate pathway as a whole must remain intact, and requires unimpaired flux through the OGC.

In islets, suppression of the OGC also reduced both GSIS and AASIS, indicating that the OGC plays a role in insulin secretion *in vivo*; however, the magnitude of the effect was less than what was seen in 832/13 cells. Greater reduction in GSIS in islets also required the simultaneous suppression of the dicarboxylate carrier. Based on these results, it is likely that islets retain the metabolic flexibility to bypass mandatory flux through the OGC, alternatively increasing flux through other mitochondrial carriers including the DIC or the oxodicarboxylate carrier (ODC), which is also capable of transporting 2OG (157). Additional research is needed to determine the contribution of these components to insulin secretion.

Also unique to islets was the observation that dimethyl-2OG failed to stimulate second-phase insulin secretion. Especially intriguing is the fact that lower concentrations of dm-2OG (~2mM) were more effective at stimulating insulin secretion than higher concentrations (12mM), which would explain some of the conflicting data in the literature reporting that dm-2OG is not a fuel secretagogue (158).

Our initial hypothesis to explain these data was that direct stimulation of islets with supra-physiological levels of dm-2OG resulted in a rapid decrease in the levels of the amino acid aspartate. Aspartate (along with asparagine, which is indistinguishable by mass spectrometry) is one of the few metabolites observed to decrease in β-cells during normal GSIS; therefore, it is possible that β-cells must build-up their levels of aspartate prior to stimulation with fuel secretagogues. Aspartate and 2OG are both substrates for the GOT1 and GOT2 enzymes, and may work in combination; Malaisse-Lagae, et al, previously reported that although L-asparagine failed to stimulate insulin secretion by itself, it caused a dose-dependent increase in secretion in the presence of leucine, BCH, or α-ketoisocaproate (KIC, a transamination product of leucine) (159), all of which are closely involved in the production of 2OG. Furthermore, it has been known for some time that our insulin-secreting cell lines must be cultured in a relatively high concentration of glutamine (2mM), in order to retain robust insulin secretion. Under these conditions, glutamine is likely converted into glutamate, which in turn might then be used to drive the production of aspartate through the GOT2 or GOT1 reactions. However, although removal of glutamine from the cell culture media for 24 hours reduced dm-2OG-SIS in 832/13 cells, it failed to reduce AASIS, which was presumed to also require 2OG flux through the GOT2 reaction, using aspartate. Furthermore, providing asparagine to islets during dm-2OG-SIS failed to substantially increase their secretion responsiveness. Additional investigation of the relationship between aspartate metabolism and GSIS, AASIS, and dm-2OG-SIS is therefore warranted.

Finally, one of the key findings of this study was that both the OGC and ICDc appear to be part of a common pathway required for insulin secretion by multiple fuel secretagogues, including glucose, glutamine, and dimethyl-2OG. However, the question remains: what is the coupling factor(s) being produced by flux through this pathway?

Multiple lines of evidence have suggested that NADPH may function as a signaling molecule in the β-cell. Here, a significant reduction in glucose-stimulated NADPH production was observed after OGC suppression, which supports previous observations with ICDc and CIC (109; 114). However, no such changes in glutamine-stimulated NADPH production were observed after OGC or ICDc suppression. Instead, under normal conditions substantially less NADPH was produced during AASIS as compared to GSIS, even though insulin secretion was the same for both secretagogues. These data cast some doubt on the universal necessity of NADPH production for all forms of fuel-stimulated insulin secretion.

Alternatively, 2OG, which is also produced by ICDc in the pyruvate-isocitrate cycling reactions, has also been suggested as a potential coupling factor in insulin secretion. While 2OG can be metabolized to succinate within the mitochondria, succinate itself does not stimulate insulin secretion (in mouse islets) (132). Furthermore, in BTBR mice, which are insulin resistant and hyperinsulinemic, but not in B6 mice (which are non-hyperinsulinemic), KIC (which is linked to 2OG production through a reaction involving glutamate) elicits a dramatic insulin secretory response. The explanation for the this is that BTBR islets have a much higher glutamate content, and are therefore more effective at generating 2OG; when islets from both mice are stimulated with dimethyl glutamate plus KIC, which should make both groups equally-effective at producing 2OG, insulin secretion (normalized to content) is the same (116).

In another study, 2.7mM glipizide (which is used to close all ATP-sensitive K+ channels) unexpectedly abolished the increase in NADPH:NADP+ ratio after stimulation with glucose or KIC; however, here KIC, but not glucose, still stimulated insulin secretion even though both secretagogues elevated calcium. Similarly, dimethyl-2OG also stimulated insulin secretion in the presence of glipizide, suggesting a potential 2OG-signaling role beyond simply being a product of the NADPH-generating ICDc reaction. Interestingly, in the presence of glipizide, dimethyl-2OG only stimulated a monophasic increase in insulin secretion in islets (although there was no indication of direct interaction of cytosolic 2OG on K_{ATP}-channels), while glutamine + BCH stimulated a biphasic increase, and glutamine alone failed to stimulated insulin release (160). These data reveal that different secretagogues and different contexts of stimulation share both common and divergent metabolic pathways regulating insulin release, some of which may rely on production of 2OG.

A potential mechanism for 2OG regulation of insulin secretion in the β-cell is as a substrate for the α-ketoglutarate hydroxylases. These enzymes can target hypoxia-inducible transcription factors (HIFs) promoting degredation and inactivation, and leading to enhanced oxidative metabolism through suppression of HIF-regulated genes (161). However, the time course for such effects would be presumed to require a longer period than the 2-hour incubations used in our experiments; furthermore, our results indicate that a signaling role for 2OG cannot be the only critical event, since generation of either cytosolic or mitochondrial 2OG still failed to rescue insulin secretion after suppression of either the OGC or ICDc.

Despite the presence of a shared pathway of fuel-stimulated insulin secretion involving flux through ICDc and the OGC, these data call into question the suggestion of NADPH or 2OG as sole signaling molecules in insulin secretion, and reveal that the pyruvate-isocitrate cycling pathway as a whole must remain intact for appropriate stimulus-secretion coupling. This observation implies that substrate cycling through this pathway may produce either alternative or complimentary signaling molecules, which may work through shared or unique mechanisms. Although a common metabolic pathway for fuel-stimulated insulin secretion has been identified, the precise identity of the coupling factors generated remains unknown.

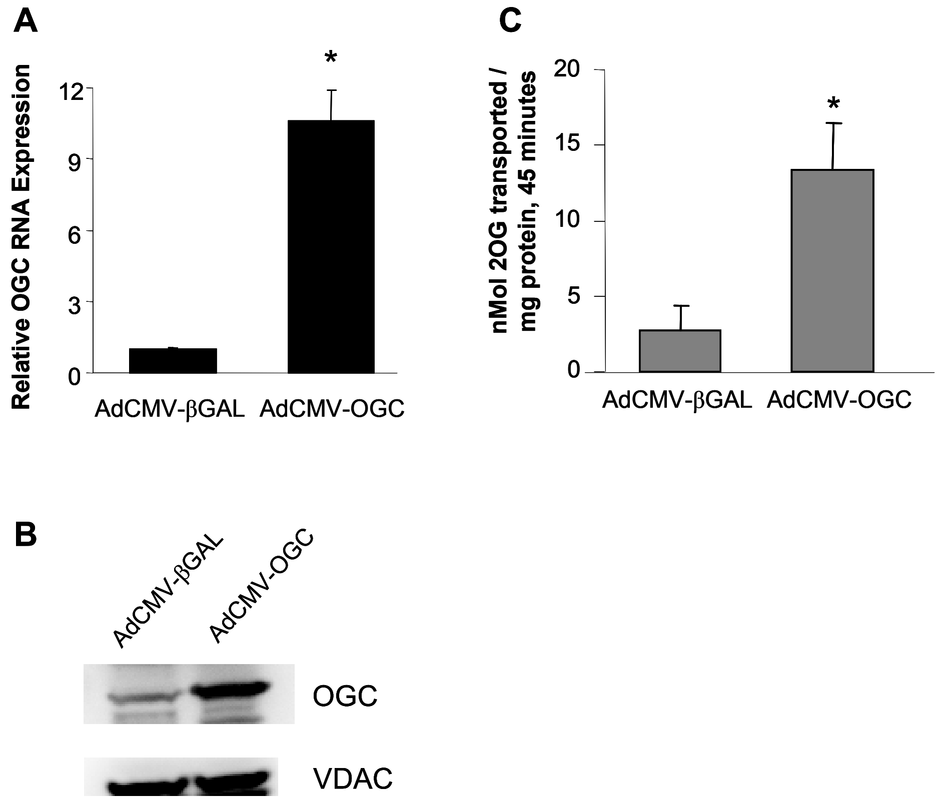

Figure 5: Overexpression of the 2-oxoglutarate carrier in 832/13 cells

The 2-oxoglutarate carrier was overexpressed in 832/13 insulinoma cells using adenovirus transduction. **(A)** Relative expression levels of the OGC as compared to control cells (ADCMV-βGAL), measured by realtime PCR. **(B)** Representative western blot of OGC protein expression for AdCMV- βGAL cells and AdCMV-OGC cells; expression levels of VDAC are shown as a loading control. **(C)** OGC activity for adCMV-βGAL cells and AdCMV-OGC cells. Data for **(A)** and **(C)** represent the mean ± SEM for 3 and 4 independent experiments, respectively. *p < 0.05

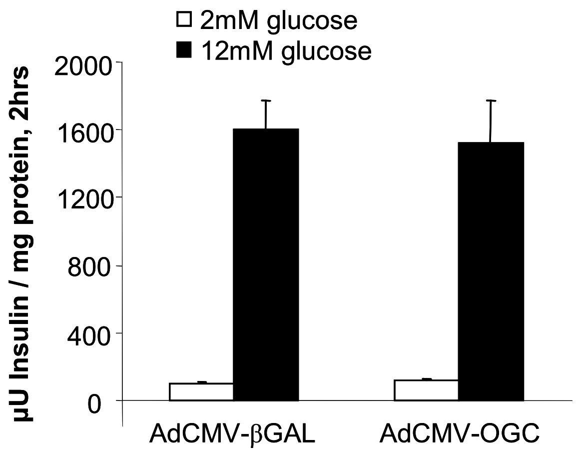

Figure 6: Effects of AdCMV-OGC on GSIS in 832/13 cells

The 2-oxoglutarate carrier was overexpressed (AdCMV-OGC) in 832/13 β-cells, and the effects on GSIS were compared with overexpression of β-galactosidase (AdCMV-βGAL). Data represent the mean ± SEM of three independent experiments.

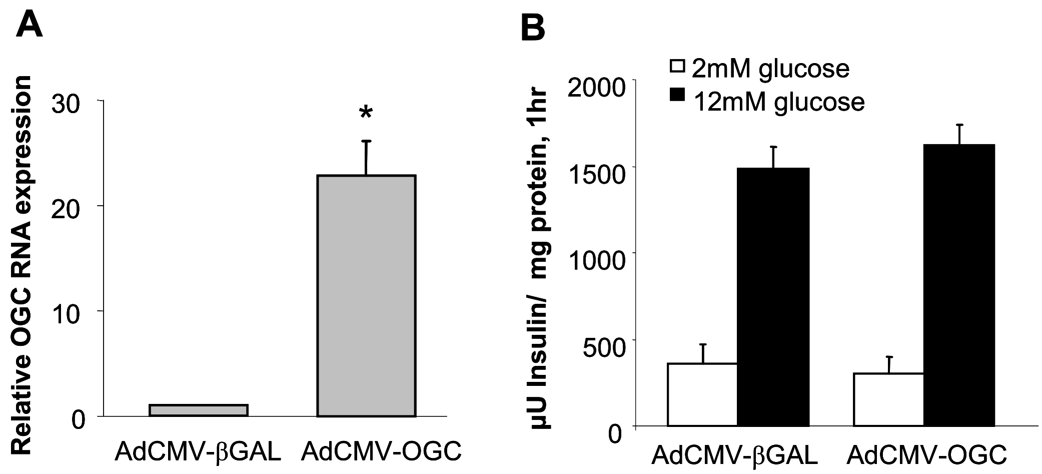

Figure 7: Effects of AdCMV-OGC on GSIS in islets

The 2-oxoglutarate carrier was overexpressed in isolated rat islets using AdCMV-OGC. **(A)** RNA expression levels of the OGC were determined relative to islets treated with AdCMV-βGAL using quantitative realtime PCR. **(B)** GSIS was measured by RIA after stimulation with low or high glucose. Data represent the mean ± SEM for 3 independent experiments. *$p<0.05$

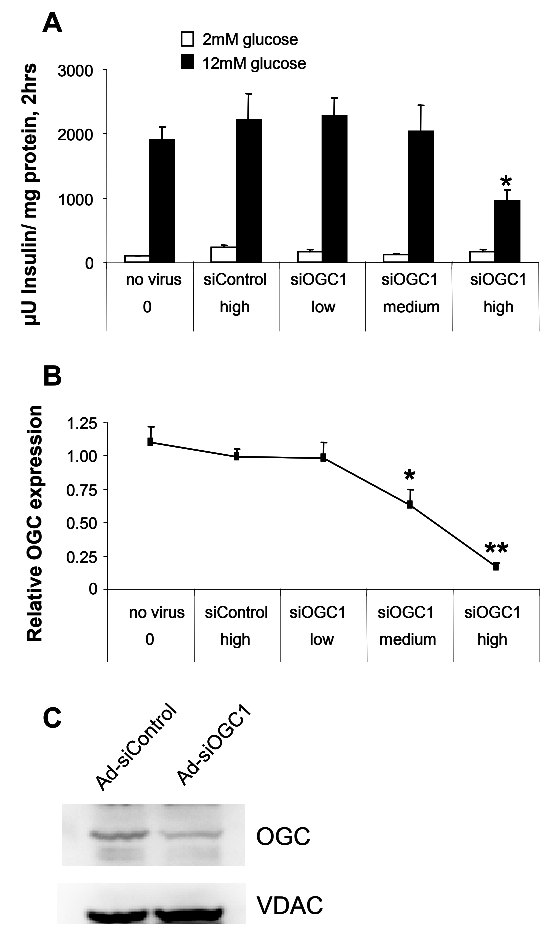

Figure 8: Effects of OGC suppression on GSIS in 832/13 cells, using Ad-siOGC1

The OGC was suppressed in 832/13 cells using Ad-siOGC1 in a dose-dependent manner. **(A)** Increasing amounts (low, medium, high) of Ad-siOGC1 dose-dependently reduced GSIS relative to both a no virus control, and a scrambled siRNA virus control, Ad-siControl. **(B)** The effects of Ad-siOGC1 on GSIS corresponded to decreased expression of OGC RNA. **(C)** At the highest dose, Ad-siOGC1 decreased OGC protein expression. Data represent the mean ± SEM for 3 independent experiments. *$p<0.05$, **$p<0.01$

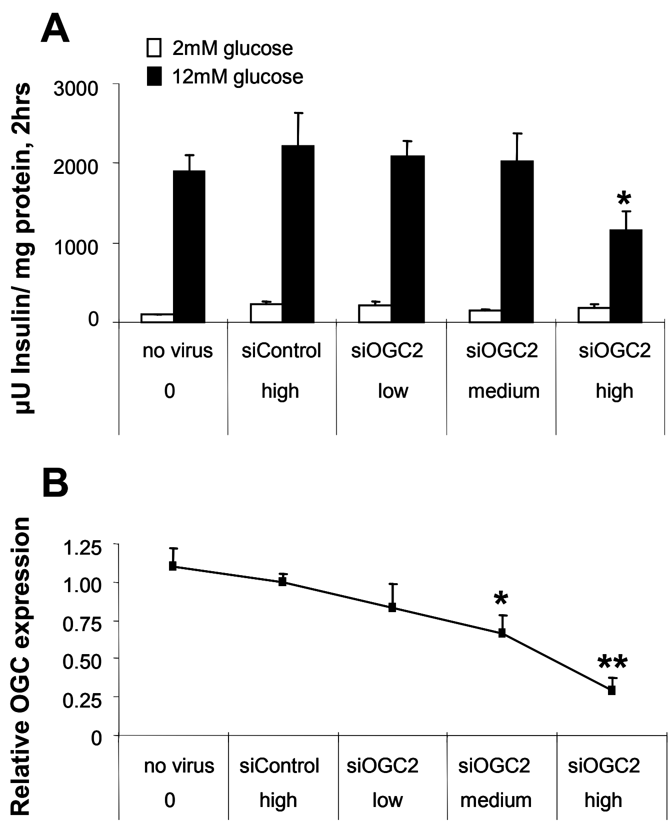

Figure 9: Effects of OGC suppression on GSIS in 832/13 cells, using Ad-siOGC2

The 2-oxoglutarate carrier was also suppressed in 832/13 cells using Ad-siOGC2, which targeted a different region of the OGC RNA transcript than Ad-siOGC1. **(A)** Similar to Ad-siOGC1, increasing amounts (low, medium, high) of Ad-siOGC2 also dose-dependently reduced GSIS relative to the no virus and Ad-siControl treatments. **(B)** OGC RNA expression was significantly decreased by treatment with Ad-siOGC1, in a dose-dependent manner. Data represent the mean ± SEM for 3 independent experiments. *p<0.05, **p<0.01

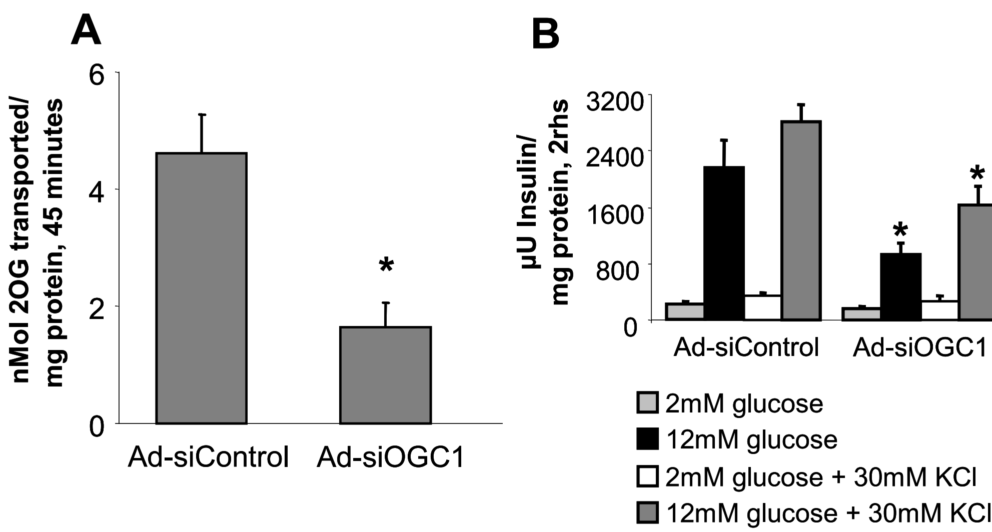

Figure 10: Effects of OGC suppression on 2OG transport and KCl-stimulated insulin secretion

The OGC was suppressed in 832/13 cells using Ad-siOGC1, and 2OG transport and KCl-stimulated insulin secretion were measured. **(A)** 2OG transport across the mitochondria was measured using an *in vitro* assay. **(B)** KCl-stimulated insulin secretion was determined in the presence of low or high glucose. Data represent the mean ± SEM of 4 or 3 independent experiments, respectively. Comparisons were made between Ad-siControl and Ad-siOGC1 for each stimulation condition. *p<0.05

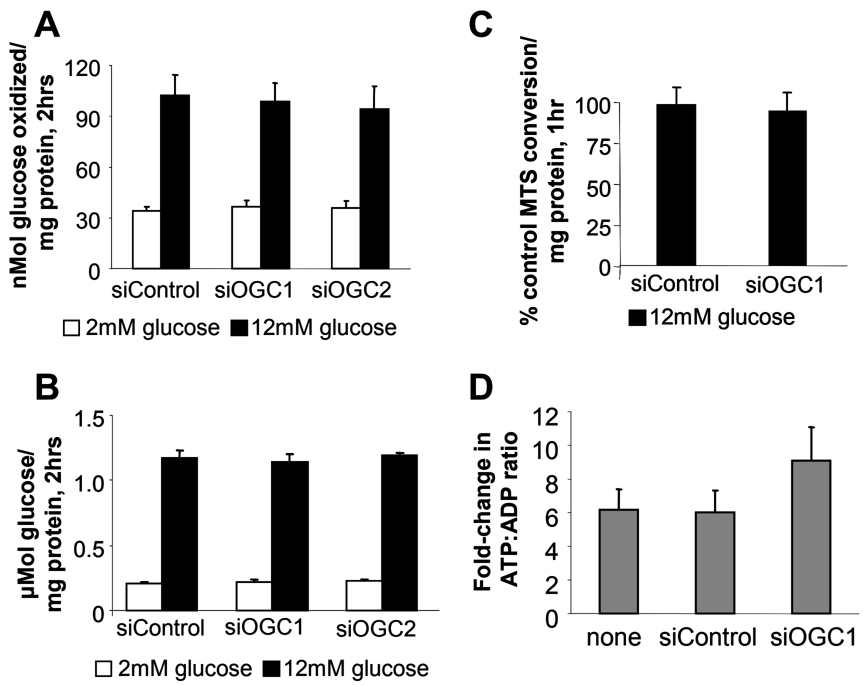

Figure 11: Effects of OGC suppression on glucose oxidation, glucose usage, cell viability (MTS conversion), and ATP production

The OGC was suppressed in 832/13 cells, and the effects on a number of metabolic parameters were determined. **(A)** Glucose oxidation and **(B)** glucose usage (glycolytic flux) were measured after treatment with Ad-siOGC1 or Ad-siOGC2, relative to Ad-siControl. **(C)** Cell viability was determined through measurements of MTS conversion, after treatment with Ad-siOGC1. **(D)** The fold-change increase in the ATP:ADP ratio after stimulation with glucose was measured after treatment with Ad-siOGC1, and compared with a no virus control and the Ad-siControl. Data represent the mean ± SEM of 4, 3, 3, and 3 independent experiments, respectively. There were no significant differences between any of the groups tested.

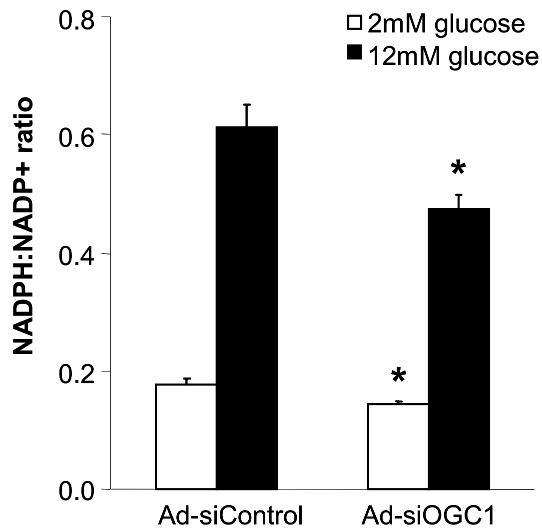

Figure 12: Effects of OGC suppression on NADPH production

The OGC was suppressed in 832/13 cells using Ad-siOGC1, and the effects on the NADPH:NADP+ ratio before and after glucose stimulation were determined. Data represent the mean ± SEM of 4 independent experiments. *$p<0.05$

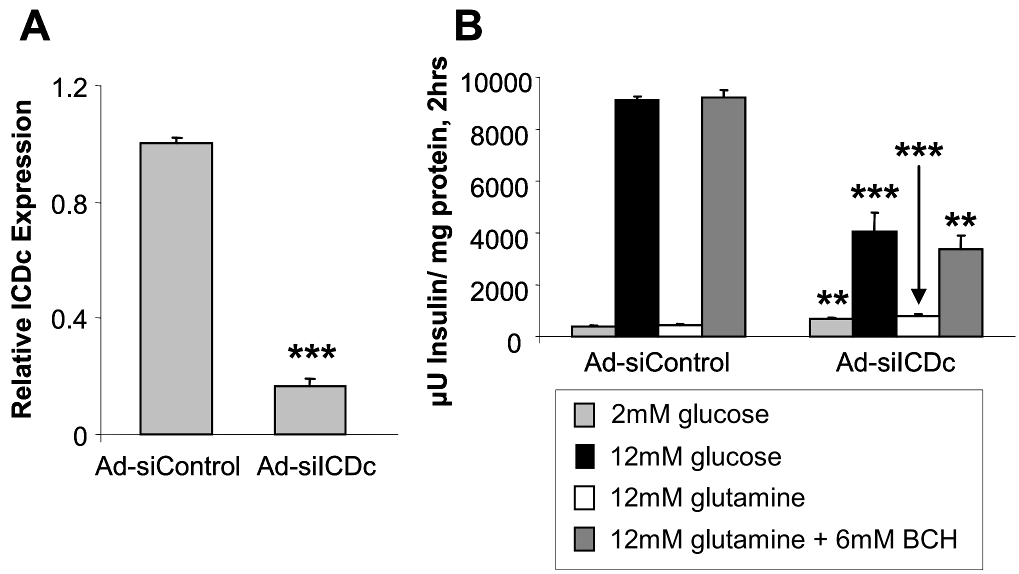

Figure 13: Effects of ICDc suppression on GSIS and AASIS

Insulin secretion in response to glucose (GSIS) or the amino acid glutamine (AASIS) was measured after suppression of the cytosolic NADP-dependent enzyme isocitrate dehydrogenase (ICDc). **(A)** Expression levels of ICDc RNA after treatment with virus Ad-siICDc, relative to the control virus Ad-siControl. **(B)** GSIS and AASIS after ICDc suppression. Data represent the mean ± SEM of 3 independent experiments. Comparisons were made between Ad-siControl and Ad-siICDc for each stimulation condition. **$p<0.01$, ***$p<0.001$

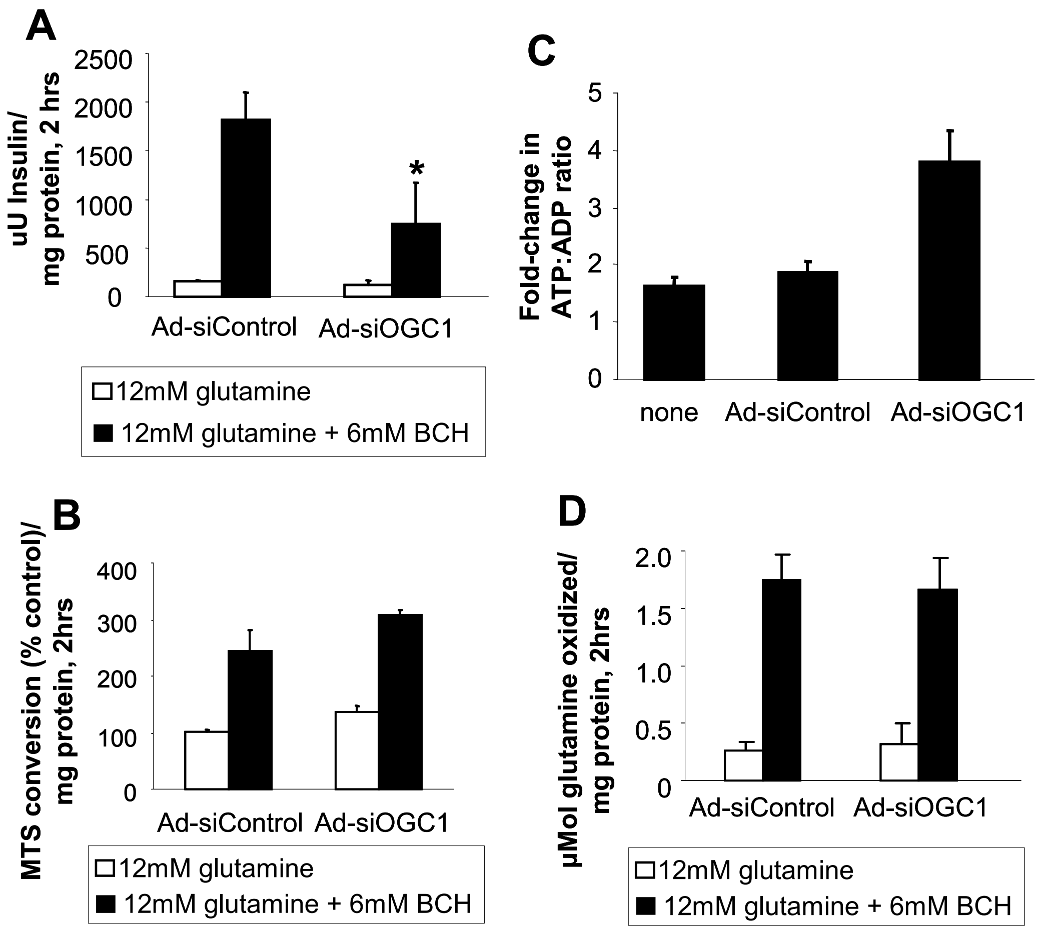

Figure 14: Effects of OGC suppression on AASIS, cell viability and ATP production after glutamine stimulation, and glutamine oxidation

The effects of OGC suppression on **(A)** glutamine-stimulated insulin secretion, **(B)** cell viability (MTS conversion) after stimulation with glutamine, **(C)** the fold change increase in the ATP:ADP ratio during AASIS, **(D)** and glutamine oxidation were measured. Data represent the mean ± SEM of 8, 3, 3, and 3 independent experiments, respectively.

*$p<0.05$

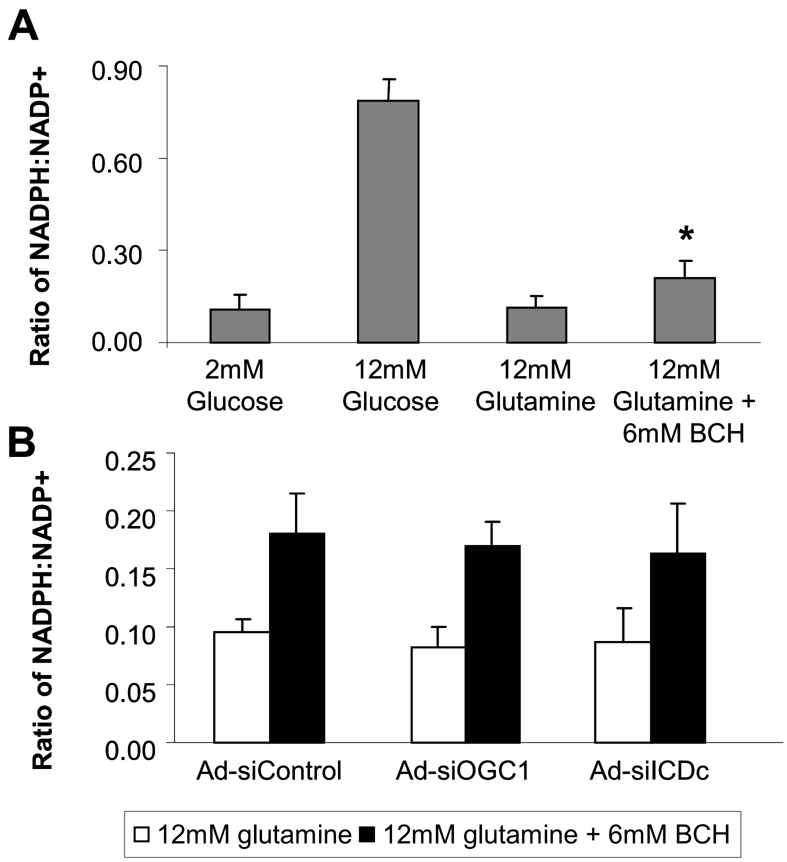

Figure 15: NADPH production during GSIS and AASIS, and effects of OGC and ICDc suppression during AASIS

The effects of OGC suppression and ICDc suppression on NADPH production during glutamine-stimulated insulin secretion were determined. **(A)** Comparison of NADPH production (ratio of NADPH:NADP+) in 832/13 β-cells stimulated with low glucose, high glucose, glutamine alone, and glutamine + BCH. **(B)** Comparison of NADPH production during AASIS for Ad-siControl, Ad-siOGC1, and Ad-siICDc treatments. Data represent the mean ± SEM for 3 independent experiments. *p<0.05

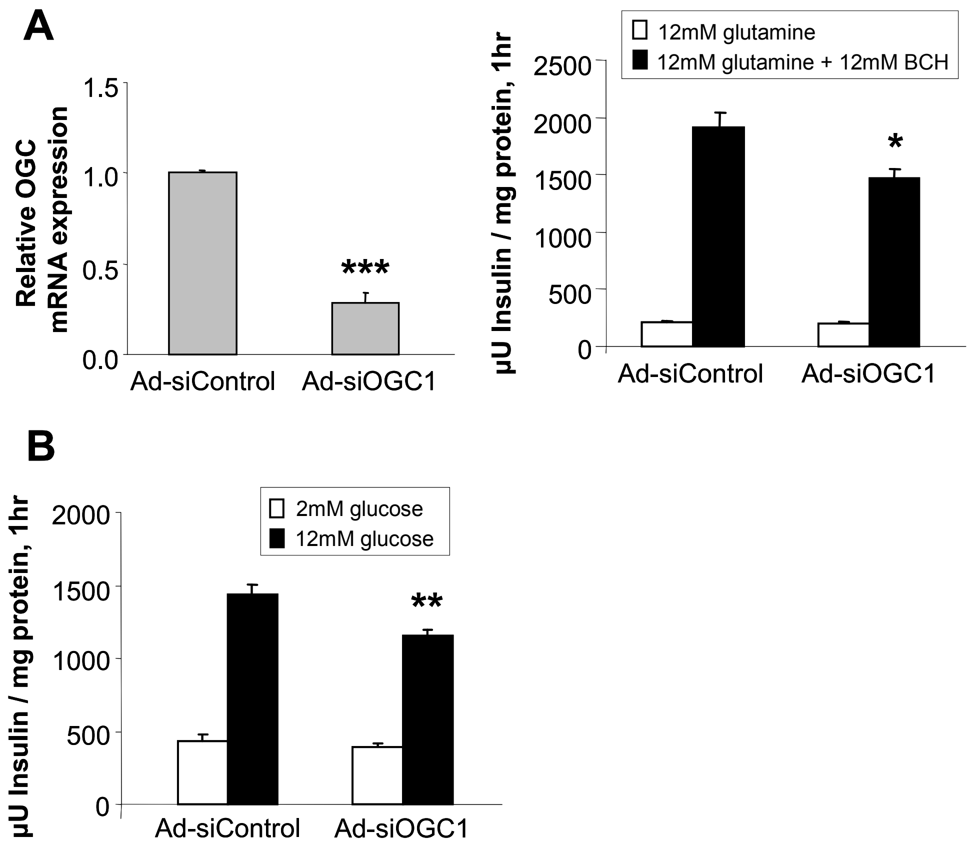

Figure 16: Suppression of the OGC in isolated rat pancreatic islets

The OGC was suppressed using Ad-siOGC1 in isolated rat pancreatic islets, and the effects on GSIS and AASIS were observed. **(A)** Expression of OGC RNA after treatment with Ad-siOGC1, relative to Ad-siControl. **(B)** GSIS and **(C)** AASIS in islets after OGC suppression. Data represent the mean ± SEM of 5, 5, and 4 independent experiments, respectively. *p<0.05, **p<0.01, ***p<0.001

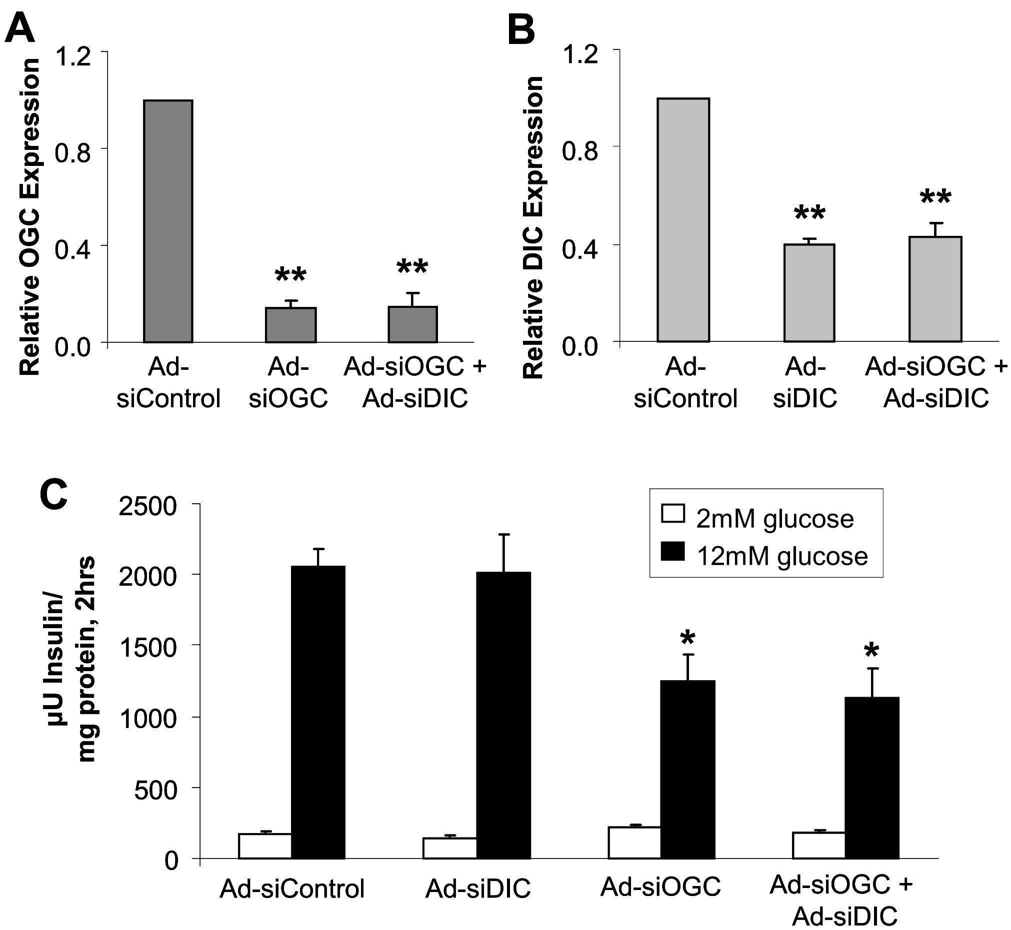

Figure 17: Suppression of the OGC and DIC in 832/13 cells

The 2-oxoglutarate carrier (OGC) and the dicarboxylate carrier (DIC) were independently and simulataneously suppressed in 832/13 β-cells. **(A)** OGC RNA expression and **(B)** DIC RNA expression after treatment of cells with Ad-siOGC or Ad-siDIC, relative to Ad-siControl. **(C)** GSIS after treatment with Ad-siControl, Ad-siDIC alone, Ad-siOGC alone, or Ad-siDIC + Ad-siOGC together. Data represent the mean ± SEM of 3 independent experiments. $*p<0.05$, $**p<0.001$

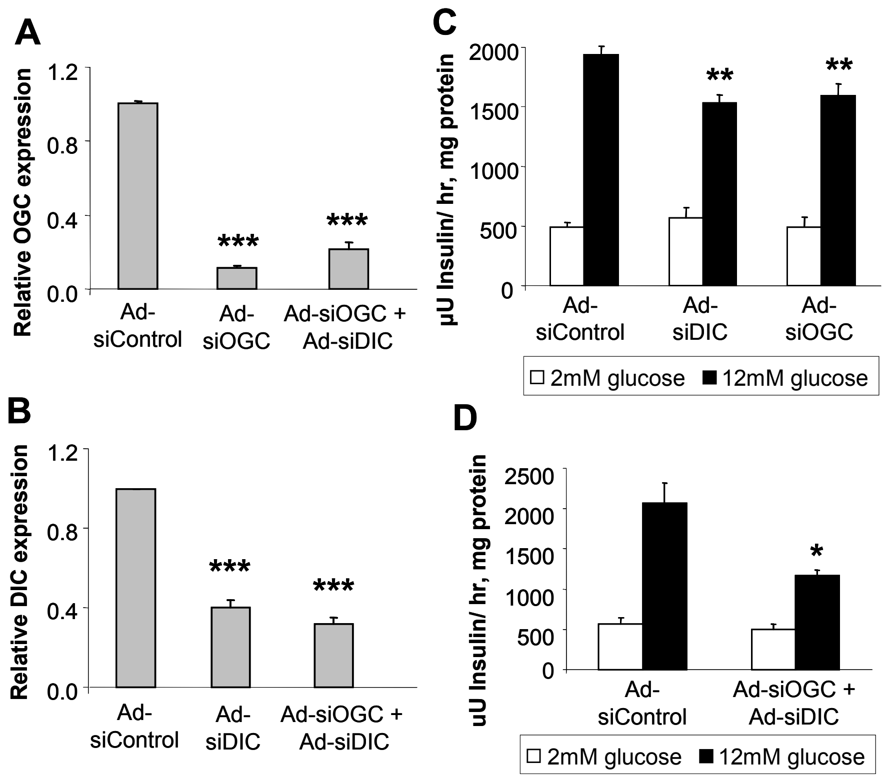

Figure 18: Suppression of the OGC and DIC in islets

The OGC and DC were independently and simultaneously suppressed in isolated rat pancreatic islets. **(A)** OGC RNA expression after treatment with Ad-siOGC and Ad-siOGC + Ad-siDIC, and **(B)** DC RNA expression after treatment with Ad-siDIC and Ad-siOGC + Ad-siDIC, relative to Ad-siControl. **(C)** GSIS after treatment with Ad-siOGC or Ad-siDIC alone. **(D)** GSIS after Ad-siOGC + Ad-siDIC together; Ad-siControl treatment was matched for virus titer. Data represent the mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001

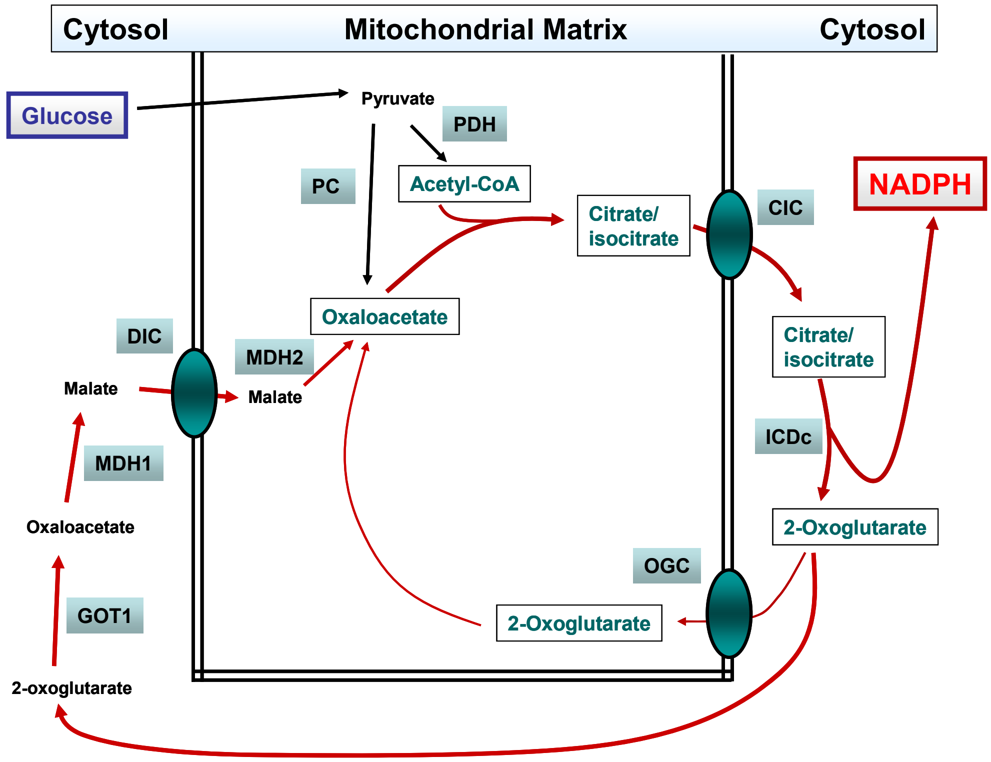

Figure 19: Potential alternative route of 2OG cycling in islets

Diagram showing an alternative route of 2OG-cycling in islets, bypassing mandatory flux through the OGC. Here, cytosolic 2OG is first converted into oxaloacetate (OAA) through the GOT1 transaminase. However, since mammalian mitochondrial do not contain transporters for OAA, conversion into malate by malate dehydrogenase (MDH1) must occur. Malate can then be transported into the mitochondrial through the DIC.

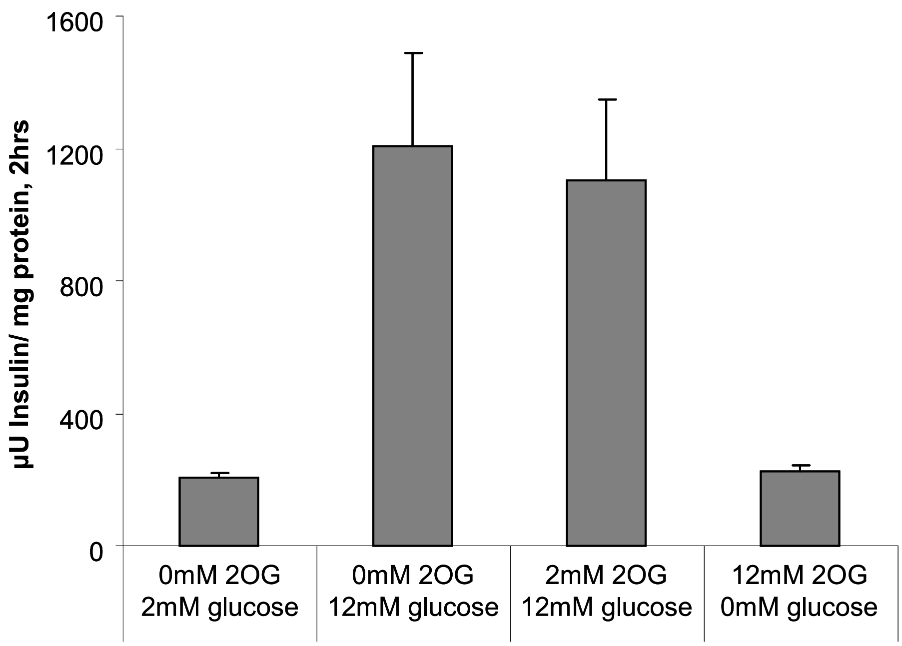

Figure 20: Insulin secretion after stimulation with the sodium-salt of 2OG

The effects of the sodium-salt of 2OG (ss-2OG) on insulin secretion were measured from untreated 832/13 β-cells. The stimulation conditions included: (bar 1) no ss-2OG, 2mM glucose, (bar 2) no ss-2OG, 12mM glucose, (bar 3) 2mM ss-2OG + 12mM glucose, (bar 4) 12mM ss-2OG, no glucose. Data represent the mean ± SEM for 3 independent experiments.

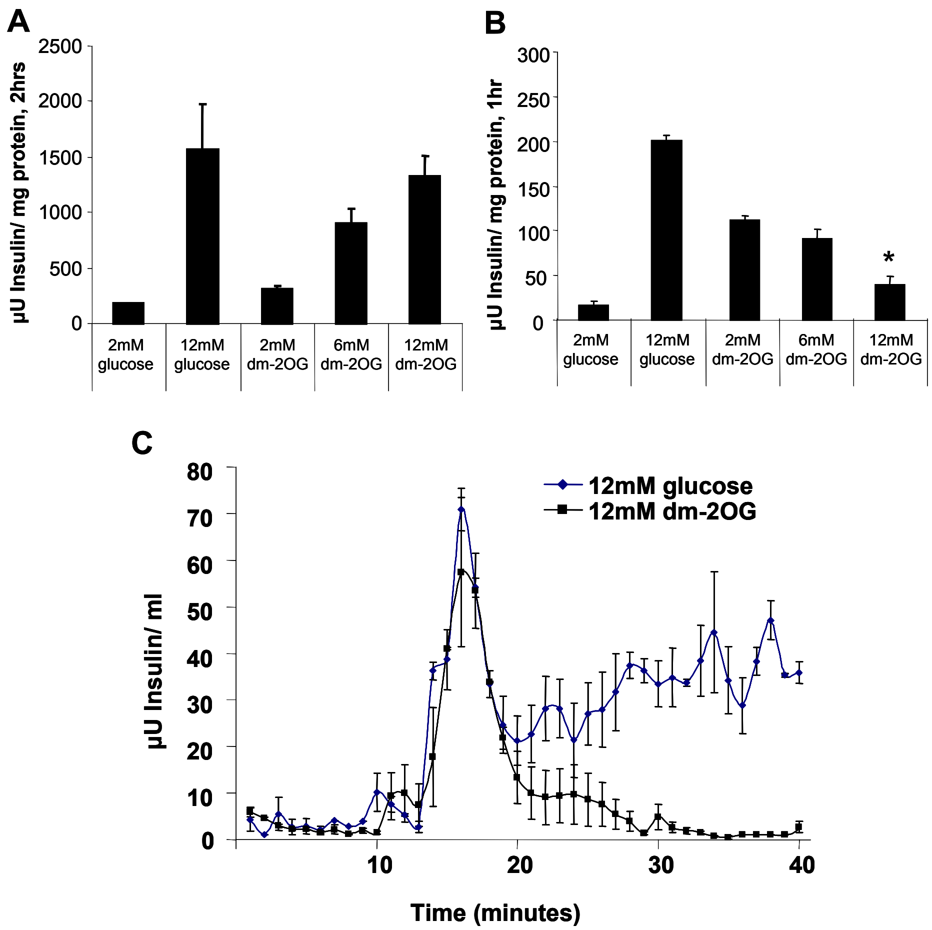

Figure 21: Effects of dm-2OG on insulin secretion from 832/13 β-cells and isolated rat islets

The stimulatory effects of dimethyl-2-oxoglutarate (dm-2OG) on insulin secretion from 832/13 β-cells and isolated islets were determined. **(A)** Dose-dependent effects of dm-2OG alone on insulin secretion from 832/13 β-cells, and **(B)** isolated islets. **(C)** Insulin secretion from rat islets perifused with either 12mM glucose alone or 12mM dm-2OG. Data represent the mean ± SEM of 3 independent experiments. *p<0.05

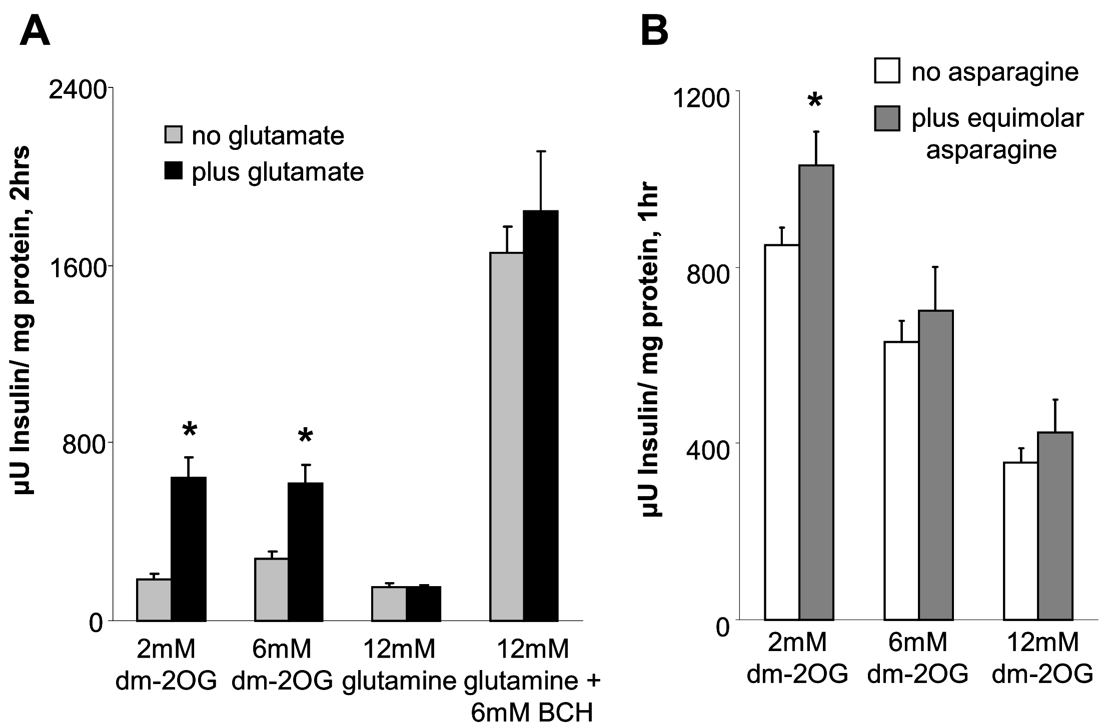

Figure 22: Effects of 24hr culture in glutamate (832/13 cells) or asparagine (islets) on dm-2OG-stimulated insulin secretion

The effects of 24hr culture of 832/13 cells with or without 2mM glutamate or isolated rat pancreatic islets with or without equimolar asparagine were determined on dm-2OG-stimulated insulin secretion. **(A)** 832/13 β-cells were cultured for 24hr in the presence or absence of 2mM glutamate. Then insulin secretion in repsonse to 2mM, 6mM dm-2OG, 12mM glutamine and 12mM glutamine + 6mM BCH was measured. **(B)** Isolated islets were cultured for 24hr in 0mM, 2mM, 6mM, or 12mM asparagine, and dm-2OG-stimulated insulin secretion was measured. Data represent the mean ± SEM of 3 independent experiments. *$p<0.05$

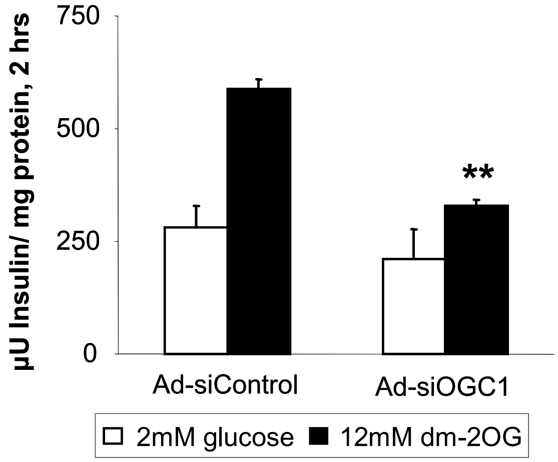

Figure 23: Effects of OGC suppression on dm-2OG-stimulated insulin secretion

The effects of OGC suppression on dm-2OG-stimulated insulin secretion were determined in 832/13 β-cells. OGC expression was reduced using Ad-siOGC1, and insulin secretion in response to 2mM glucose (basal conditions) or 12mM dm-2OG (stimulatory conditions) was measured. Data represent the mean ± SEM for 3 independent experiments. **$p<0.01$

4. Glutamate Dehydrogenase Plays a Necessary Role in Both Glucose- and Glutamine-Stimulated Insulin Secretion

Glutamate dehydrogenase (Glud1) activity is allosterically regulated by a wide range of factors in animals, and plays a key role in metabolism in pancreatic β-cells. Activation of Glud1 is a requirement for insulin secretion in response to glutamine, but the metabolic signals necessary for the full secretion response remain unknown. Therefore, we investigated the role of Glud1 in glutamine-(amino acid) stimulated insulin secretion (AASIS) as well as glucose-stimulated insulin secretion (GSIS). Adenovirus-mediated siRNA suppression of Glud1 in clonal pancreatic beta-cells (832/13) and isolated rat islets resulted in a significant decrease in both AASIS and GSIS. The effect on AASIS was attributed to a significant decrease in glutamine oxidation, but no changes in glucose usage or glucose oxidation were observed after Glud1 suppression during GSIS in 832/13 cells. Instead, the ratio of NADPH:NADP+ was decreased during GSIS. Interestingly, the increase in glutamate/ glutamine levels usually observed during GSIS was also decreased by Glud1 suppression. These data indicate that Glud1 plays a critical role in both AASIS and GSIS, but likely by affecting different aspects of the secretory mechanism for each fuel source.

4.1 Introduction

Insulin secretion from pancreatic β-cells occurs in response to increased metabolism of fuel substrate, including glucose and glutamine. While generation of ATP is known to be necessary for triggering insulin release, much less is known regarding the ATP-independent signals responsible for long-term potentiation of secretion (152).

Neonatal hyperinsulinism (HI) is a potentially life-threatening disease characterized by excessive release of insulin and elevated plasma ammonia levels (hyperammonia, HA) (137). HI/HA patients are hyper-responsive to leucine, and are susceptible to hypoglycemia following high-protein meals (162). An important link has been demonstrated in these patients between excessive insulin secretion and activating mutations in the enzyme glutamate dehydrogenase (Glud1) (137) that lead to dysregulation and loss of inhibition to GTP or ATP (163).

In humans, only a single species of Glud1 cDNA has been found so far, coded by 13 exons, but DNA southern and cytogenic analyses indicate the presence of a human Glud gene family: pseudogenes Gludp2-5; 2 and 3 are truncated forms of Glud1 containing exons 2-4; Gludp4 and 5 are similar to the 3′ part of Glud1 cDNA and were likely generated by retrotransposition. All human Glud pseudogenes 2-5 have diverged recently in evolution (164), but it is unclear what role, if any, these play in physiology and metabolism.

Glud1, however, plays a very important role in metabolism. It is localized to the inner mitochondrial matrix, and catalyzes the reversible deamination of L-glutamate to 2-oxoglutarate using NADP+ as the coenzyme (165). Although Glud1 is thought to normally be inhibited in the non-stimulatory state, insulin release is known to be regulated by a number of factors that also modify activity of Glud1 (166), and multiple lines of evidence reveal an important role for Glud1 in insulin secretion.

Activation of Glud1 by leucine or the non-hydrolyzable leucine analogue BCH (beta-2-aminobicyclo(2.2.1)-heptane-2-carboxylic acid) is a prerequisite for insulin secretion in response to glutamine, which by itself fails to stimulate insulin release from normal β-cells despite being converted into glutamate (167). Ultimately, the stimulatory effects of glutamine on the β-cell are directly correlated with increased glutamine oxidation, which is only possible after activation of Glud1 allows glutamine-derived carbon to enter the TCA cycle (as 2OG).

Overexpression in Cos-7 cells of the Glud1 H266C mutation identified from HI/HA syndrome severely impairs allosteric inhibition of Glud1 by ATP and GTP, while Min6 cells expressing this Glud1 mutant demonstrate elevated insulin secretion under low glucose conditions and hypersecretion of insulin in response to glutamine alone (168). Additionally, β-cell-specific overexpression of a different Glud1 mutant, H454Y, in transgenic mice further confirmed the HI/HA hypoglycemia and insulin secretion phenotype (169). Together, these data confirm that activation of Glud1 leads to increased insulin release.

Conversely, allosteric inhibition of Glud1 using epigallocatechin gallate (EGCG), a polyphenol compound found in green tea, has been reported to decrease insulin secretion (153); however, additional data presented in this dissertation calls into question the use of EGCG as a Glud1-specific inhibitor. Instead, more conclusive data showing detrimental effects of loss of Glud1 in regulating insulin secretion has been demonstrated using islets from Glud1-/- mice. Here, absence of glutamate dehydrogenase in β-cells led to reduced GSIS, which was able to be restored by re-expression of Glud1 (170).

The studies just cited from other laboratories strongly suggest an important role for Glud1 in control of insulin secretion, but do not provide insight into the metabolic pathways or stimulus/secretion coupling factors underlying these effects. For these reasons, we have chosen to further investigate the role of Glud1 in insulin secretion in response to different fuel secretagogues (glucose, glutamine, and dm-2OG). To this end, we measured the effects of Glud1 suppression on cell viability, substrate oxidation, NADPH, and changes in amino acid levels after fuel stimulation. Our results reveal that suppression of Glud1 fails to alter insulin secretion in response to dm-2OG, but instead decreases secretion after stimulation with either glucose or glutamine. Although the effect on AASIS was most easily attributed to reduced glutamine oxidation, no changes in glucose usage or glucose oxidation were observed during GSIS. Instead, suppression of Glud1 decreased glucose-stimulated production of NADPH, while also reducing the increase in glutamate/ glutamine levels normally observed after stimulation with glucose. Taken together, these results indicate that although flux through Glud1 plays a necessary role in insulin secretion in response to two of the most potent fuel secretagogues (glutamine and glucose), the effects of Glud1 suppression on insulin secretion are mediated through different metabolic mechanisms with respect to these two fuels.

4.2 Results

Overexpression of Glud1 fails to alter insulin secretion

Wild-type Glud1 was cloned by Danhong Lu (Newgard Lab) and used to generate a Glud1 overexpression adenovirus, AdCMV-Glud1. Transduction of 832/13 cells with AdCMV-Glud1 increased Glud1 mRNA and protein expression levels, and Glud1 activity (as measured in vitro) (Fig. 24a-c). However, overexpression of wild-type Glud1 in 832/13 β-cells failed to alter either glutamine-stimulated insulin secretion (AASIS) (Fig. 24d) or glucose-stimulated insulin secretion (GSIS). Glud1 was also overexpressed in isolated primary rat islets. Again, although Glud1 transcript levels were increased by AdCMV-Glud1, no changes in insulin secretion were observed (Fig. 25a,b).

Inhibition of Glud1 using EGCG

Based on the findings of Li, et al, the compound EGCG was used to inhibit Glud1 in 832/13 β-cells, and the effects on insulin secretion and metabolism were measured. Although acute treatment with EGCG resulted in a dramatic decrease in both AASIS and GSIS (Fig. 26a,b), the decrease in AASIS was accompanied by no change in glutamine oxidation (Fig. 26c), which was troubling considering flux of glutamate into the TCA cycle as 2OG would be expected to be decreased through the use of a Glud1 inhibitor. Whereas no changes in glucose oxidation or glucose usage were observed during GSIS (Fig. 26d,e), treatment with EGCG severely decreased insulin secretion in response to high glucose or stimulatory glutamine + KCl (Fig. 26a,b). This result seemed to indicate that in addition to inhibiting Glud1, EGCG may have off-target or non-specific effects on the β-cell. Therefore, additional experiments were conducted using siRNA-mediated adenovirus transduction techniques.

Suppression of Glud1 by siRNA leads to reduced AASIS

Two siRNA adenoviruses, Ad-siGlud1-1 and Ad-siGlud1-2, were generated to target two separate unique regions of the Glud1 transcript. Treatment of 832/13 β-cells with Ad-Glud1-1 or Ad-Glud1-2 resulted in a decrease in Glud1 RNA (Fig. 27a), as well as a decrease in protein expression and Glud1 activity (in vitro assay) (Fig. 28a,b).

Suppression of Glud1 using either virus decreased insulin secretion during stimulation with 12mM glutamine + 6mM BCH in the presence or absence of depolarizing concentrations of KCl, but as expected, had no effect on insulin secretion under basal conditions (12mM glutamine) with or without KCl (Fig. 27b). MTS conversion, as a measurement of cell viability and mitochondrial function, was unaffected by suppression of Glud1 during AASIS (Fig. 28c). Instead, suppression of Glud1 resulted in a significant decrease in glutamine oxidation (Fig. 28d).

Suppression of Glud1 leads to reduced GSIS

In addition to the effects on AASIS, suppression of Glud1 also reduced insulin secretion during stimulation by high glucose with or without KCl, while basal insulin secretion at low glucose with or without KCl was unaffected (Fig. 29a). Cell viability, glucose usage and glucose oxidation were also not altered by Glud1 suppression (Fig. 29b-d).

Glud1 suppression reduces NADPH production during GSIS, but not AASIS

Suppression of Glud1 reduced NADPH production during GSIS (Fig. 30a), similar to what was observed after OGC and ICDc suppression. However, likely because NADPH levels were already much lower under stimulatory conditions during AASIS as compared to GSIS, no further decrease in NADPH levels was observed after suppression of Glud1 during AASIS (Fig. 30b).

Effects of Glud1 suppression on the levels of glutamate/ glutamine

Metabolic profiling of amino acid levels was performed by tandem mass spectrometry. Suppression of Glud1 blunted the increase in glu* levels normally observed after stimulation with glucose (Fig. 31a), while reducing the fall in glu* levels observed during AASIS, likely by reducing the oxidation of glutamate by decreasing entry into the TCA cycle as 2OG (Fig. 31b).

Interestingly, insulin secretion in response to stimulation with dm-2OG alone was unaffected by Glud1 suppression (Fig. 31c), in contrast to impaired insulin secretion under the same conditions in response to suppression of OGC or ICDc. Thus, Glud1 is necessary for GSIS, but not for stimulation by a metabolite from the pyruvate-isocitrate cycle. This serves as further evidence for the existence of multiple signals generated by fuel metabolism in control of insulin secretion.

Exogenous supply of dm-2OG fails to rescue AASIS or GSIS

Based on the idea that suppression of Glud1 decreases conversion of glutamate to 2OG, dimethyl-2OG was used in an attempt to rescue both AASIS and GSIS by exogenously providing the product of the Glud1 reaction. However, exogenous supply of two different concentrations of dm-2OG (2mM and 6mM) failed to rescue either AASIS or GSIS after Glud1 suppression (Fig. 32a,b). This result is consistent with the finding that Glud1 suppression does not impair secretion in response to dm-2OG, and provides additional evidence of multiple pathways of control of insulin secretion.

Both GSIS and AASIS are reduced in primary cells by Glud1 suppression

To verify that the observed effects of Glud1 suppression on insulin secretion applied to primary tissue, Glud1 was also suppressed in isolated rat islets, and the effects on AASIS and GSIS were measured. Here, reduced expression of Glud1 caused impairment of insulin secretion, regardless of whether the fuel source was glutamine or glucose (Fig. 33b,c).

4.3 Discussion

Multiple lines of evidence have revealed that glutamate dehydrogenase plays a key role in regulating fuel-stimulated insulin secretion from pancreatic β-cells, but the precise mechanism remains unknown. In this study, we used pharmacologic and genetic manipulations to alter Glud1 expression and activity, and observed the corresponding effects on insulin secretion and metabolism.

Although overexpression of mutant forms of Glud1 has previously been shown to increase insulin secretion and responsiveness to amino acids (168; 169), here, overexpression of the wild-type form of Glud1 failed to alter insulin secretion from either 832/13 β-cells or isolated rat islets, likely because it was still able to be appropriately regulated by GTP and other factors, and in the context of these experiments was not a rate-limiting step in insulin secretion. A previous report by Carrobio, et al., showed that overexpression of Glud1 failed to alter insulin release at basal or intermediate glucose concentrations or during stimulation with 5mM glutamine alone, but increased insulin secretion in response to high glucose or glutamine plus BCH, which corresponded to increases in glutamine metabolism (171). Therefore, it is possible that in some experimental contexts, flux through Glud1 can be a rate-limiting step in secretion, although the factors responsible for these differences remain unknown.

Pharmacologic inhibition of Glud1 using EGCG decreased AASIS as well as GSIS, which was not observed to change in the study by Li, et al (153). However, the severity of effects on insulin secretion and lack of change in glutamine oxidation rates suggested potential off-target effects. Indeed, a search of the literature revealed that EGCG has been shown to affect a wide range of molecular processes including cancer cell growth and apoptosis (172; 173), NO signaling and cell migration (174), and telomerase and DNA methyltransferase activity (175), among others, making the effects of EGCG on insulin secretion difficult to interpret. Therefore, siRNA suppression techniques were used in place of EGCG for further investigation of Glud1.

Suppression of Glud1 by adenovirus-mediated siRNA transduction resulted in a decrease in both AASIS and GSIS, although in contrast with EGCG insulin secretion was not completely impaired. The effects of molecular suppression of Glud1 seemed to require a metabolic signal, as no effects were observed on non-metabolic insulin secretion machinery, measured with a depolarizing concentration of KCl under basal substrate conditions (low glucose or glutamine with no added BCH). These effects were similar to the observations from the Glud1-/- mouse, which demonstrated that Glud1 activity appears to account for ~40% of the insulin secretory response. In islets from these animals, both first- and second-phase insulin secretion were reported to be reduced, although glucose-stimulated ATP generation was not decreased. Interestingly, Glud1-/- mice still maintained glucose homeostasis under normo-caloric conditions, revealing that in the absence of insulin resistance, maximal secretory capacity was not required (170).

The decrease in AASIS after Glud1 suppression was attributed to reduced Glud1-derived 2OG entry into oxidation in the TCA cycle. In support of this idea, metabolic profiling of amino acid levels revealed that Glud1 suppression reduced the drop in glu* levels normally observed after stimulation with BCH, which suggests decreased glutamate flux into 2OG (and likewise decreased oxidation in the TCA cycle). Within this context, cells stimulated with dimethyl-2OG would have substrate available for oxidation that was being supplied downstream of the Glud1 reaction; therefore, it is not surprising that Glud1 suppression failed to decrease dm-2OG-SIS.

Although Glud1 suppression did reduce GSIS, this effect did not correspond to decreased glucose usage or decreased glucose oxidation. Instead, similar to previous studies, we measured reduced NADPH production after suppression of Glud1. These data provide more supporting evidence of a necessary role for NADPH in regulating glucose-stimulated insulin secretion.

Somewhat unexpectedly, supplementation with exogenous dm-2OG failed to rescue either GSIS or AASIS in cells with suppressed Glud1 suppression, despite the fact that stimulation with 12mM dm-2OG alone was unaffected. One possible explanation, based on our previous observations in islets, is that the compound dm-2OG may not be a perfect substitute for endogenously-produced 2OG, which would likely be generated in a specific cellular comparent at a highly-regulated rate. It is important to note that dm-2OG by itself fails to stimulate insulin secretion to the levels observed with glucose or glutamine. To that extent, dm-2OG may simply not be capable of triggering full, robust insulin secretion, and is therefore not able to rescue defects in 2OG production after Glud1 suppression.

An alternative explanation is that flux through Glud1 flows in the reverse direction during GSIS (75) contributing to the increase in glutamate levels observed after glucose stimulation (176). In this case, treatment with dm-2OG would certainly fail to rescue defects in GSIS after Glud1 suppression. Our data lend some support to this theory, in that the glucose-stimulated increase in glu* levels were observed to be reduced by suppression of Glud1.

As mentioned previously in this dissertation, glutamate has been suggested to act as a coupling factor during insulin secretion, although this idea is not without controversy. The increase in glucose-stimulated glutamate levels can be prevented by overexpression of glutamate decarboxylase (GAD65), which lowers cytoplasmic glutamate levels, and this has been shown to result in impaired insulin secretion (77). Furthermore, islets from SUR1-/- mice, which were used as a model of constitutive K_{ATP}-channel closure and elevated Ca^{2+} levels, secrete more insulin in response to glutamine + BCH than control islets, and are more sensitive to amino acids and glutamine alone (45). Addition of dimethyl glutamate directly to normal β-cells during stimulation causes a left-ward shift in the concentration dependence of glucose during GSIS, although no additional increase in insulin release is seen at the highest concentration of glucose (16.7mM) (177).

Despite these observations, several reports instead make the argument that the elevation of glutamate levels and insulin secretion are not causally related. Addition of glutamine to normal islets has been shown to cause a 10-fold increase in glutamate levels but does not stimulate insulin secretion (178), and does not further increase secretion after depolarization with high K+ or sulfonylurea (79). Also, the high K_m of Glud1 for ammonia is presumed to minimize the reverse reaction of 2OG to glutamate (179), while lack of ^{15}N incorporation suggests that Glud1 does not participate in glutamate production (169). Finally, high glucose is thought to inhibit flux through Glud1 through generation of GTP. Although this would suggest the enzyme may not be active during GSIS, preliminary findings from our lab indicate that GTP levels actually decrease during GSIS, which would lead to activation, not inhibition, of Glud1.

Instead, additional evidence for glutamate as a coupling factor comes from recent studies suggesting a potential mechanism of action in the β-cell contributing to amplification of insulin secretion. The metabotropic glutamate receptors, which include eight subtypes in three groups, are G-protein coupled receptors found on the plasma membrane of the β-cell as well as on insulin containing granules (81). Brice, et al, has shown that these receptors contribute to the modulation of GSIS; specific agonists of group I (mGluR1, 5) and group 2 (mGluR2, 3) increased insulin release at both low and high glucose, whereas group 3 agonists (mGluR4, 6, 7, 8) inhibited insulin release at high glucose (180). Storto, et al, also observed that mGlu5 metabotropic glutamate control insulin secretion. Although these did not respond to extracellular agonists, they did respond to a cell-permeant analog of glutamate, resulting in increased [Ca2+] and insulin secretion. Both effects were largely attenuated by the mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP). Additionally, wild-type mice treated with the antagonist showed reduced second phase insulin secretion, while mGlu5-/- mice showed a defective insulin response after glucose pulse (81). As glutamate is stored in both insulin- and glucagon-containing granules (181), release of glutamate along with insulin during first-phase secretion could be one mechanism of amplifying and sustaining second-phase secretion.

A signaling role has also been proposed for glutamine in insulin secretion. In this regard, production of glutamate would still be important, as it would serve as the required substrate in the glutamine synthase reaction, which converts glutamate to glutamine. Glutamine, in SUR1-/- mice, is a more potent secretagogue than either leucine or dimethyl glutamate, while in normal islets, high glucose doubles glutamine levels. However, treatment with methionine sulfoximine, and inhibitor of glutamine synthetase, suppressed insulin secretion in response to a glucose ramp and increased buildup of glutamate and aspartate. This suppression was reversed by addition of either glutamine, or 6-diazo-5-oxo-L-norleucine (non-hydrolyzable leucine analogue) (78).

Ultimately, the data presented in this dissertation indicate that Glud1 plays an important role in regulating insulin secretion in response to two of the most potent fuel secretagogues, glucose and glutamine, as both AASIS and GSIS were significantly reduced by suppression of Glud1. However, these effects appear to be mediated through different mechanisms; reduced expression of Glud1 decreases AASIS by reducing fuel oxidation, but impairs GSIS by altering oxidation-independent processes, including

NADPH production and glutamate formation. Importantly, suppression of Glud1 failed to alter dm-2OG-SIS, which suggests that that dm-2OG likely stimulates secretion through a reaction pathway that does not require flux through Glud1. Therefore, while flux through Glud1 is a shared requirement for robust GSIS and AASIS but not dm-2OG-SIS, it remains to be determined whether glucose, glutamine, and dm-2OG regulate secretion through the generation of shared or unique coupling factors.

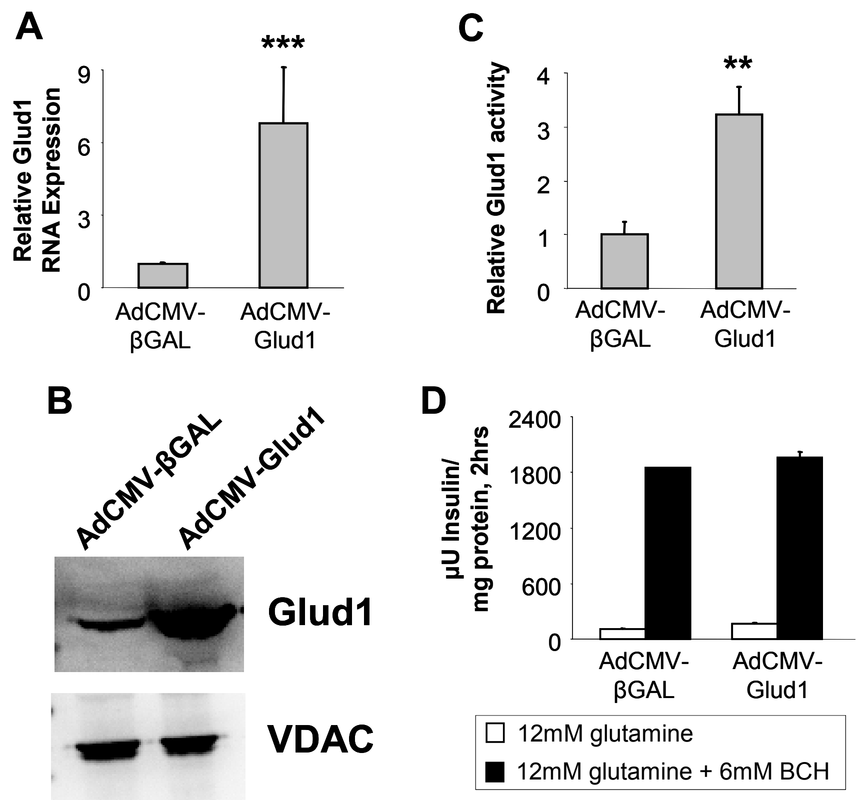

Figure 24: Overexpression of wild-type Glud1 in 832/13 β-cells

Glutamate dehydrogenase was overexpressed in 832/13 β-cells, and the effects on AASIS were measured. **(A)** Glud1 RNA expression levels after treatment with AdCMV-Glud1, relative to treatment with AdCMV-βGAL. **(B)** Representative western blot of Glud1 protein expression levels. VDAC expression is shown as a loading control. **(C)** Glud1 activity in 832/13 cells after Glud1 overexpression by AdCMV-Glud1. **(D)** Glutamine-stimulated insulin secretion after overexpression of Glud1, in 832/13 cells. Data for **(A)**, **(C)**, and **(D)** represent the mean ± SEM of 5 independent experiments. $^{**}p<0.01$, $^{***}p<0.001$

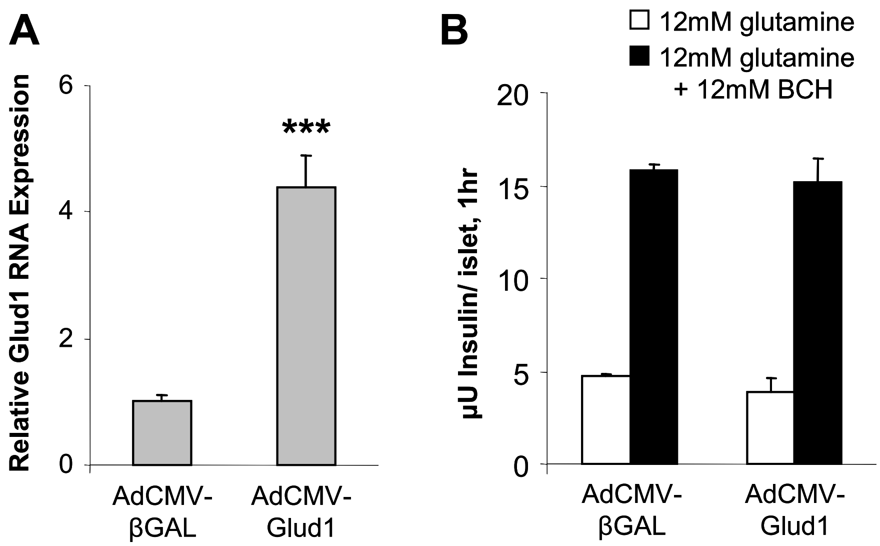

Figure 25: Glud1 overexpression in isolated rat pancreatic islets

Glud1 was overexpressed in isolated islets, and the effects on AASIS were measured. **(A)** Glud1 RNA expression, determined by realtime PCR. **(B)** Glutamine-stimulated insulin secretion. Data represent the mean ± SEM of 4 independent experiments. ***$p<0.001$

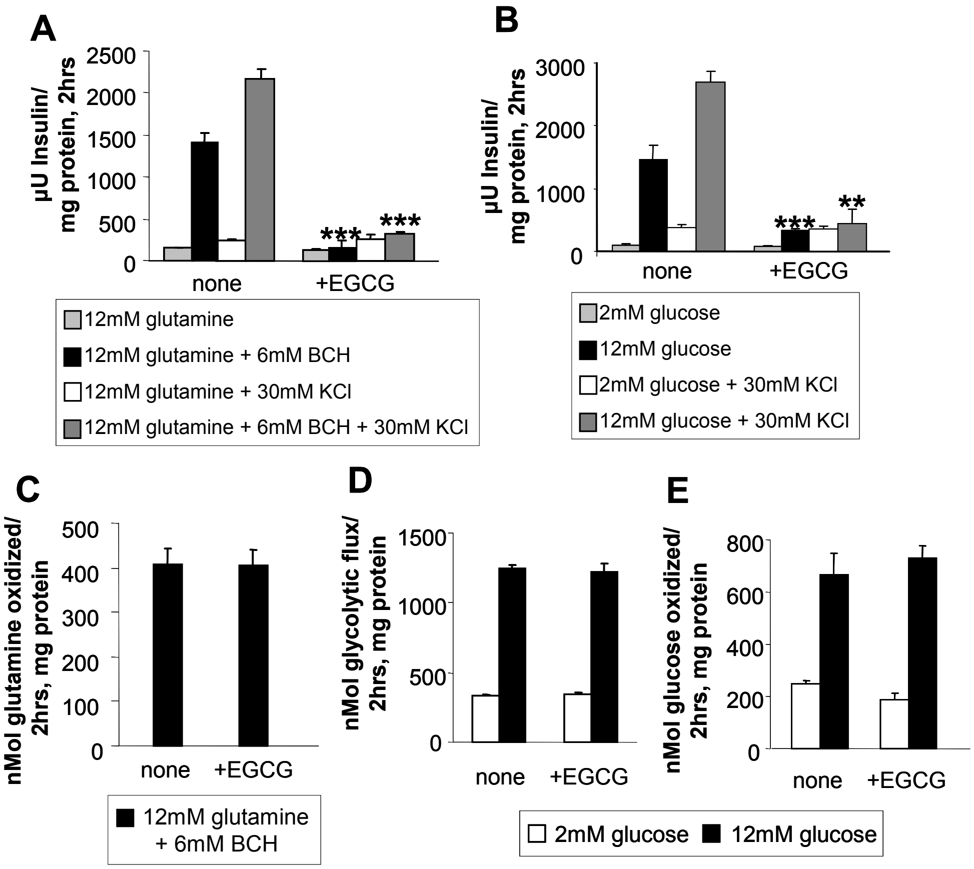

Figure 26: Effects of EGCG on insulin secretion and metabolism in 832/13 β-cells

The effects of acute treatment with 200μM epigallocatechin gallate (EGCG) were measured in 832/13 cells. **(A)** AASIS with and without KCl. **(B)** GSIS with and without KCl. **(C)** Glutamine oxidation. **(D)** Glucose usage (glycolytic flux). **(E)** Glucose oxidation. Data represent the mean ± SEM for 3 independent experiments. **p<0.01, ***p<0.001

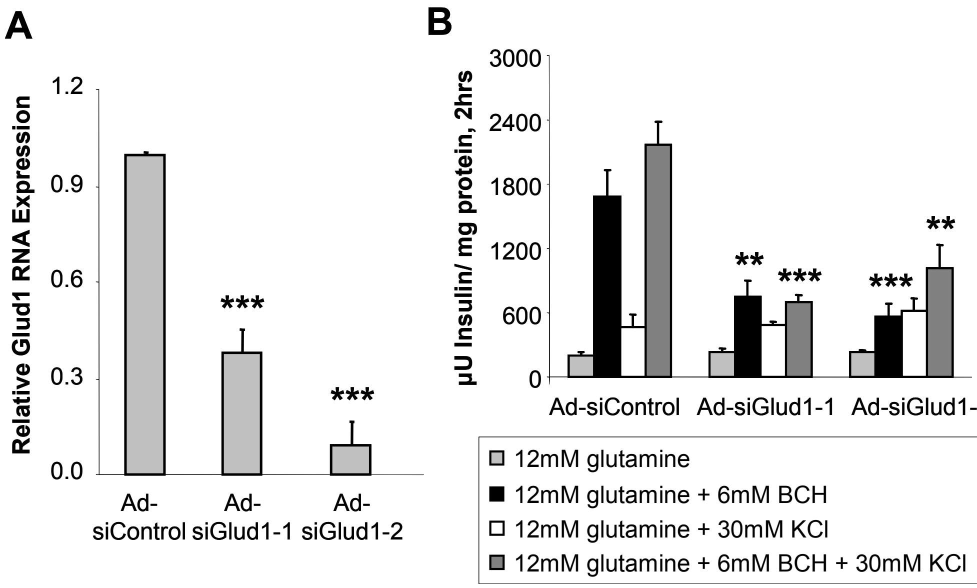

Figure 27: Effects siRNA-mediated suppression of Glud1 on AASIS

Glutamate dehydrogenase was suppressed using adenovirus in 832/13 cells, and the effects on AASIS in the presence of absence of 30mM KCl were measured. **(A)** Glud1 RNA expression levels after treatment with either Ad-siGlud1-1 or Ad-siGlud1-2, relative to Ad-siControl. **(B)** Glutamine-stimulated insulin secretion in the presence or absence of 30mM KCl. Data represent the mean ± SEM of 3 independent experiments. **p<0.01, ***p<0.001

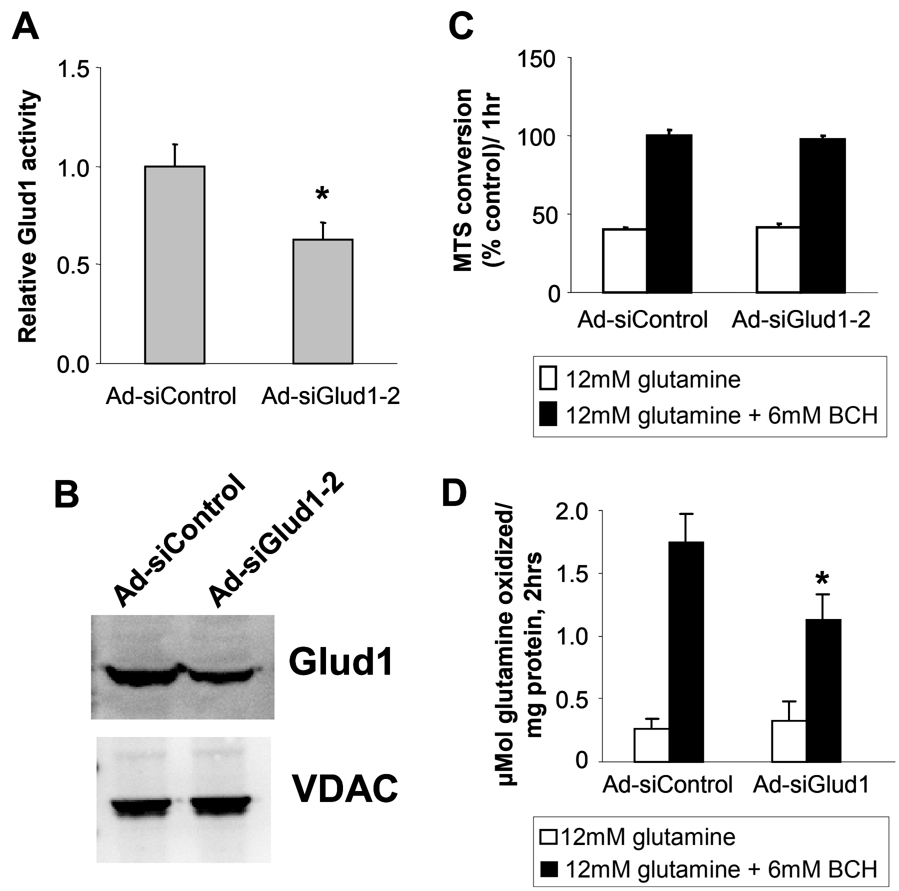

Figure 28: Effects of Glud1 suppression on activity, protein expression, cell viability, and glutamine oxidation in 832/13 β-cells

Glud1 was suppressed in 832/13 cells using Ad-siGlud1-2. **(A)** Glud1 activity as determined by *in vitro* assay, relative to Ad-siControl. **(B)** Representative western blot showing Glud1 protein expression levels after treatment with Ad-siControl or Ad-siGlud1-2. VDAC expression is shown as a loading control. **(C)** Cell viability (MTS conversion) during stimulation with glutamine alone or glutamine + BCH. **(D)** Glutamine oxidation. Data for **(A)**, **(C)**, and **(D)** represent the mean ± SEM of 4, 5, and 3 independent experiments, respectively. *p<0.05

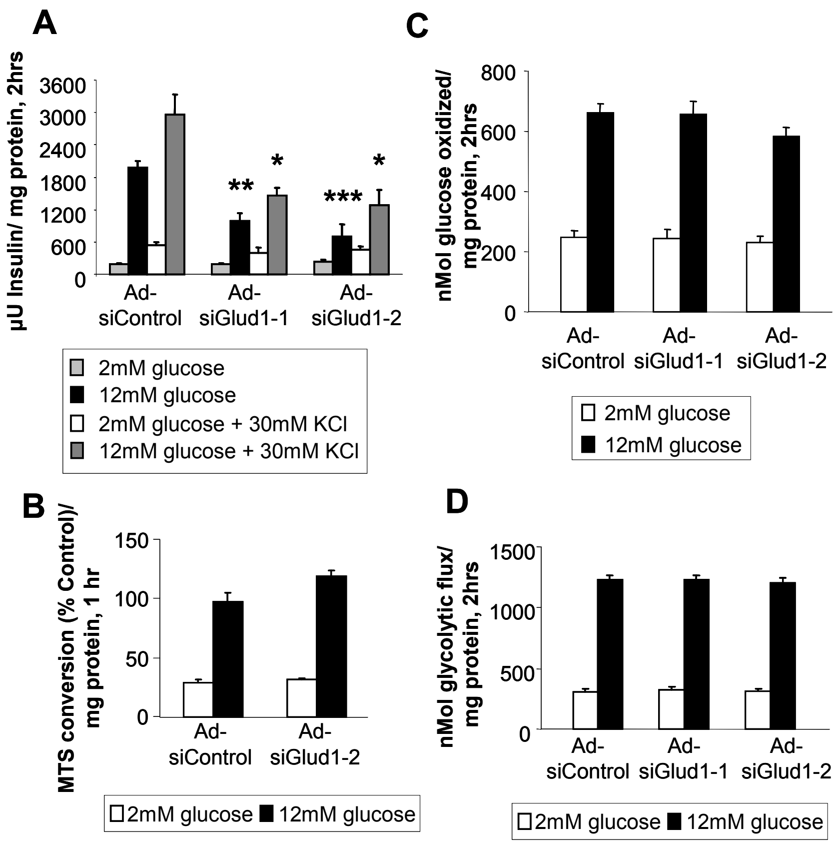

Figure 29: Effects of Glud1 suppression on GSIS +/- KCl, cell viability, glucose oxidation, and glucose usage in 832/13 β-cells

Glud1 was suppressed using Ad-siGlud1-1 and Ad-siGlud1-2 in 832/13 cells, and the effects on GSIS and glucose metabolism were measured. **(A)** GSIS in the presence of absence of 30mM KCl. **(B)** Cell viability (MTS conversion) determined after stimulation with low or high glucose. **(C)** Glucose oxidation and **(D)** glucose usage (glycolytic flux) determined during two-hour stimulation with low or high glucose. Data represent the mean ± SEM of 5, 3, 3, and 3 independent experiments, respectively. *p<0.05, **p<0.01, ***p<0.001

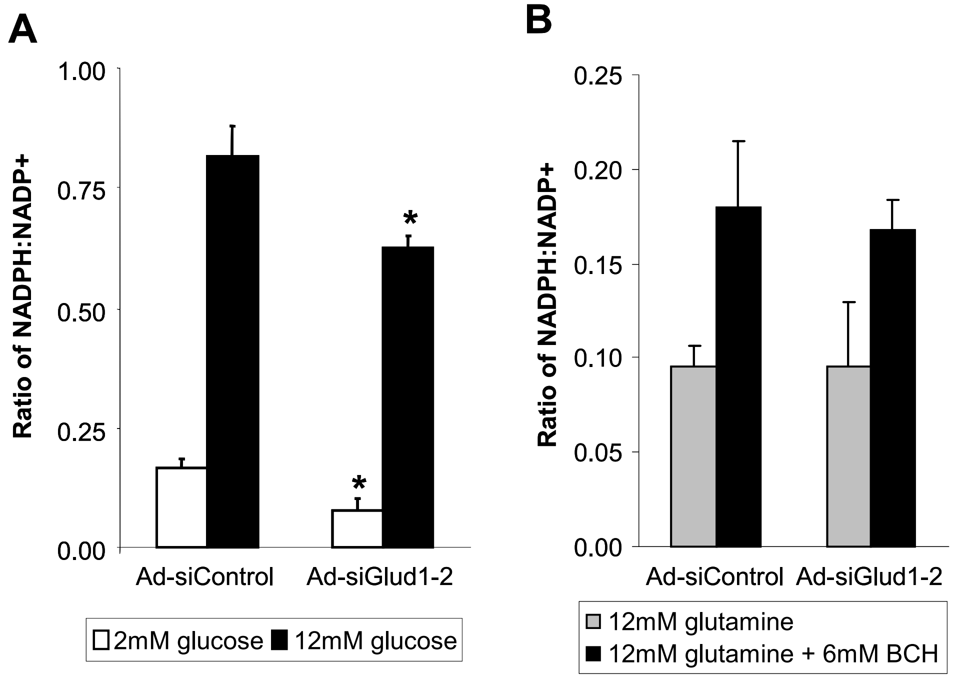

Figure 30: Effects of Glud1 suppression on NADPH production during GSIS or AASIS

Glud1 was suppressed in 832/13 β-cells using Ad-siGlud1-2, and the effects on NADPH production during **(A)** glucose-stimulated insulin secretion or **(B)** glutamine-stimulated insulin secretion were measured. Data represent the mean ± SEM of 3 independent experiments. *$p<0.05$

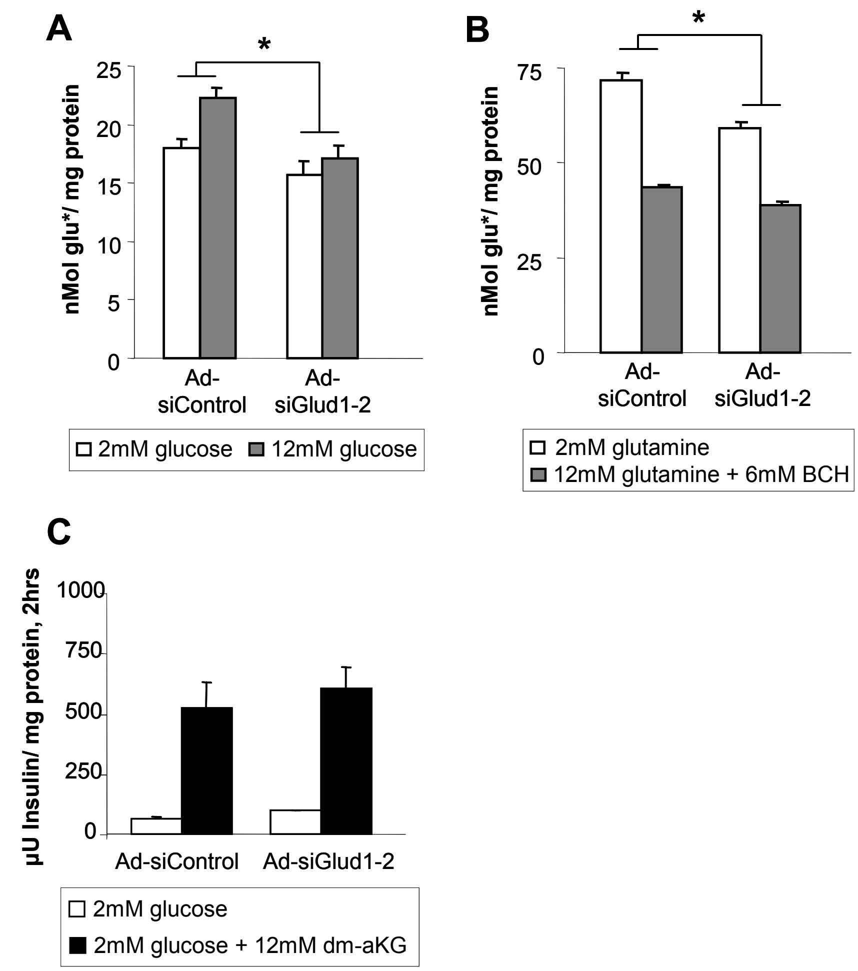

Figure 31: Amino acid profiling and dimethyl-2OG-stimulated insulin secretion after suppression of Glud1 in 832/13 β-cells

The total intracellular levels of aspartate/ asparagine (asp*) and glutamate/ glutamine (glu*) were measured after Glud1 suppression in 832/13 cells stimulated with glucose or glutamine. **(A)** Levels of glu* after stimulation with glucose. **(B)** Levels of glu* after stimulation with glutamine. **(C)** Insulin secretion in response to either 2mM glucose, or 2mM glucose + 12mM dm-2OG. Data represent the mean ± SEM of 4, 4, and 3 independent experiments, respectively. *$p<0.05$

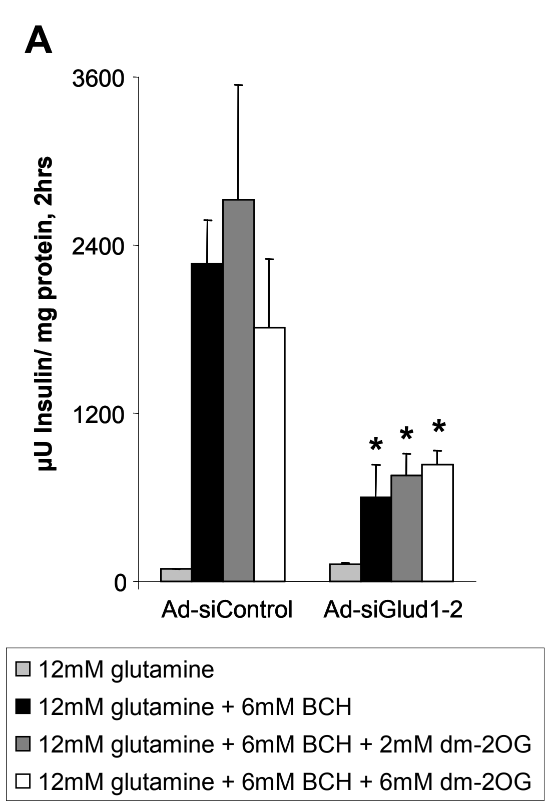

Figure 32: Addition of dm-2OG during GSIS and AASIS after Glud1 suppression

Dimethyl-2-oxoglutarate (dm-2OG) was added to cells during GSIS and AASIS after Glud1 suppression, in an attempt to rescue insulin secretion. **(A)** Addition of dm-2OG during AASIS. **(B)** Addition of dm-2OG during GSIS. Data represent the mean ± SEM of 3 independent experiments. *$p<0.05$

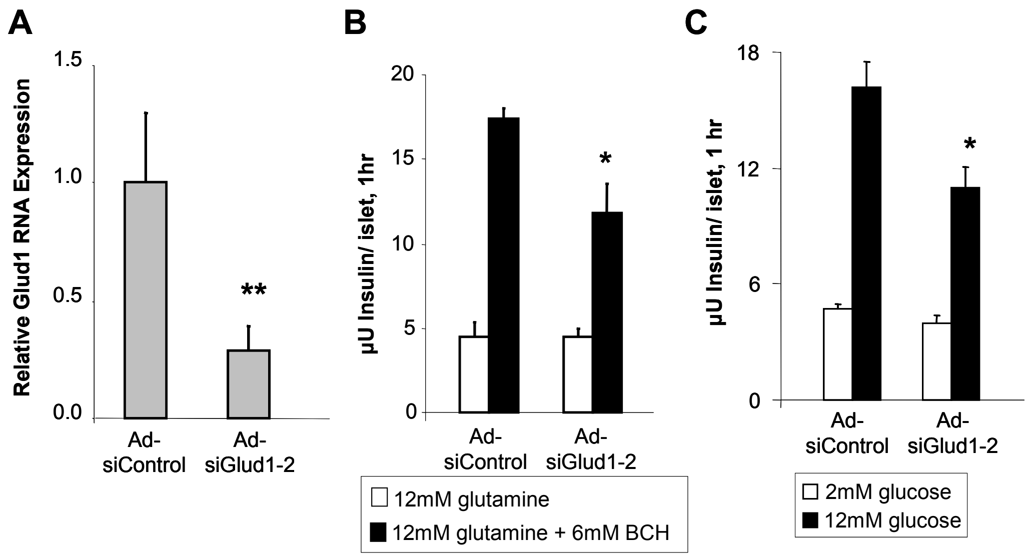

Figure 33: Effects of Glud1 suppression on GSIS and AASIS in isolated rat pancreatic islets

Glud1 was suppressed in isolated islets using Ad-siGlud1-2, and the effects on GSIS and AASIS were measured. **(A)** Glud1 RNA expression determined by quantitative realtime PCR, relative to treatment with Ad-siControl. **(B)** Glutamine-stimulated insulin secretion. **(C)** Glucose-stimulated insulin secretion. Data represent the mean ± SEM of 4 independent experiments. $*p<0.05$, $**p<0.01$

5. Conclusions and Future Directions

The rapid increase in worldwide rates of obesity over the past several decades has been accompanied by a corresponding increase in the incidence of type 2 diabetes. Although many obese individuals are able to compensate for increased insulin resistance through increased production and release of insulin, in many cases impaired glucose-sensing and β-cell failure mark the transition from mild-hyperglycemia and pre-diabetes to full-blown type 2 diabetes. Complications of unregulated hyperglycemia include blindness, renal failure, and reduced circulation to the extremities, often requiring amputation (182). Therefore, the development of better treatment therapies to reverse β-cell dysfunction and correct hyperglycemia is absolutely essential.

The process of glucose-stimulated insulin secretion from pancreatic β-cells occurs in response to glucose metabolism, and involves both ATP-dependent and ATP-independent phases. The first phase of insulin secretion is thought to be triggered by ATP-dependent events, and involves β-cell depolarization and release of intracellular Ca^{2+} stores. The second phase of insulin secretion involves generation of ATP-independent coupling factors, which act to augment and amplify secretion through both Ca^{2+}-dependent and Ca^{2+}-independent signaling, thereby sustaining insulin release (48). While first-phase insulin secretion occurs for only 10-15 minutes after stimulation with glucose, second-phase can occur over several hours, and is responsible for up to 70% of the total insulin released (54). Therefore, identification of the second messenger coupling factors responsible for second-phase secretion would be most beneficial for the development of diabetic treatment strategies that target insulin secretion.

Previous work by our lab and others suggested a strong link between robust, sustained insulin secretion, and flux through the pyruvate cycling reactions. Three key pieces of evidence link pyruvate cycling with GSIS: the correlation of increased flux through PC with enhanced GSIS in the immortalized β-cell lines, the increase in insulin secretion observed after stimulation with DMM (which increases flux through PC and pyruvate cycling), and the decrease in secretion observed after treatment with PAA (which inhibits flux through PC and decreases pyruvate cycling) (23). Further investigation revealed that flux through the pyruvate-isocitrate cycle (114), but not the pyruvate-citrate (111) or pyruvate-malate cycles (105), was necessary for full insulin secretion in response to glucose. However, the identity of the coupling factor(s) produced by this pathway as well as the role of this pathway in other forms of fuel-stimulated insulin secretion was not determined.

A key step in pyruvate-isocitrate cycling is the production of NADPH and 2OG by cytosolic isocitrate dehydrogenase. Both of these reaction products have been suggested to function as second messengers in the β-cell. Therefore, to further investigate the role of NADPH and 2OG metabolism in insulin release and determine if all forms of fuel-stimulated insulin secretion depend on flux through a common set of metabolic reactions, my thesis project has focused on two components downstream of the ICDc reaction: the mitochondrial 2-oxoglutarate transporter, and the enzyme glutamate dehydrogenase.

5.1 The 2-oxoglutarate carrier

The 2-oxoglutarate carrier (OGC) functions as a bi-directional anti-porter for 2OG and malate, and along with other carriers facilitates cytosolic and mitochondrial metabolism through the shuttling of metabolites across the inner mitochondrial membrane (134). To date, no β-cell pathologies have been reported to be due to variations in OGC expression or activity. Instead, the OGC was selected for investigation because it is located downstream of ICDc at a critical junction linking 2OG cytosolic and mitochondrial metabolism.

Overexpression of the carrier had no effect on GSIS in either the 832/13 β-cell line or isolated rat islets. However, suppression of the carrier reduced GSIS by greater than 50%, with no corresponding decreases in glycolytic flux (glucose usage), glucose oxidation, ATP production, or cell viability, and no decrease in KCl-stimulated insulin secretion under low glucose conditions. Instead, in the 832/13 cells there was a substantial reduction in insulin secretion in the presence of KCl and high glucose, indicating that ATP-independent metabolic signaling was altered by OGC suppression. Furthermore, NADPH production during GSIS, which has previously been correlated with insulin release (114), was observed to be decreased by suppression of the OGC.

OGC suppression also decreased insulin secretion in response to glutamine plus BCH, with no corresponding changes to glutamine oxidation, ATP-production, cell viability, or non-metabolic insulin release in response to KCl plus glutamine alone. These observations indicate that two unique fuel secretagogues having separate initial routes of metabolism, glucose and glutamine, share a common pathway of stimulating second-phase insulin secretion, and that for proper β-cell function OGC transport and the pyruvate-isocitrate cycling pathway in its entirety must remain intact.

However, in contrast to stimulation with glucose, stimulation with glutamine failed to produce an equivalent increase in the NADPH:NADP+ ratio. Furthermore, suppression of the OGC as well as suppression of ICDc failed to reduce NADPH production during AASIS, despite the fact that OGC and ICDc suppression both resulted in decreased glutamine-stimulated insulin secretion. These data therefore call into question the universal importance of NADPH generation during all forms of fuel-stimulated insulin secretion, and instead imply that the pyruvate-isocitrate cycling pathway may serve an additional or alternative purpose.

One such possibility included the production of cytosolic 2OG, so a variety of experiments were performed to study the effects of 2OG and dm-2OG on insulin secretion. In cell lines, the sodium salt of 2OG, which is not membrane permeable, failed to alter insulin release in the presence or absence of glucose, indicating that 2OG likely does not serve an extracellular signaling function. By contrast, dm-2OG was able to stimulate insulin release in a dose-dependent manner, while OGC suppression reduced dm-2OG-stimulated secretion, suggesting that insulin secretion was regulated by the actual metabolism of 2OG, and not non-metabolic signaling.

In the islets, stimulation with dm-2OG produced effects on insulin secretion that were different from the cell lines. Up to 2mM, dm-2OG stimulated insulin secretion, while higher concentrations resulted in less and less insulin release, until 12mM dm-2OG failed to stimulate release above basal conditions. Because 2OG and aspartate are both substrates for the GOT1 and GOT2 reactions, and aspartate levels are known to decrease during GSIS, several experiments were performed to try to increase aspartate levels in islets in an attempt to increase their responsiveness to 2OG. However, only a small increase in secretion was observed after overnight culture in asparagine. Similarly, removal of glutamine from the 832/13 culture media for 24hrs led to a slight decrease in insulin secretion, but this could not be precisely attributed to changes in asparagine/ aspartate concentrations. Therefore, few concrete conclusions can be drawn from these data.

Another difference between the cell lines and islets was in the magnitude of effect of OGC suppression on insulin secretion. Although suppression reduced both GSIS as well as AASIS in islets, the full effect on GSIS was only observed after simultaneous suppression of the DIC (which had no effect in the cell lines). These data suggest that islets may not require mandatory flux through the OGC for the completion of the pyruvate-isocitrate cycling pathway, and can instead rely on alternative mitochondrial carriers such as the DIC.

Taken together, these data indicate that the OGC is part of a necessary pathway for regulating insulin secretion in pancreatic β-cells in response to multiple fuel secretagogues (glucose, glutamine, and dm-2OG). However, although suppression of the OGC was shown to result in reduced GSIS and NADPH production, the exact mechanism linking reduced OGC expression to changes in AASIS or dm-2OG-SIS is still unknown.

5.2 Glutamate dehydrogenase

Glutamate dehydrogenase (Glud1) is responsible for the reversible reaction of glutamate to 2OG (plus ammonia) within the mitochondria, allowing oxidation in the TCA cycle (179). Animal Glud1 activity is regulated by a diverse range of factors and metabolites including inhibition by GTP and ATP, and activation by leucine, which is the most abundant amino acid found in protein (136). In liver, this regulation allows amino acid degradation to be suppressed when other fuels such as glucose and fats are available, but increased when protein is ingested and excess amino acids are available (136).

In β-cells, several lines of evidence have revealed that changes in Glud1 activity alter insulin secretion. Multiple factors that allosterically regulate Glud1, such as leucine, also affect insulin release (166), while the syndrome of neonatal hyperinsulinism has been linked to activating mutations in Glud1 (137) and reproduced in Glud1 H454Y transgenic mice (169). Alternatively, suppression of Glud1 has also been shown to decrease AASIS (153), while Carrobio, et al, recently reported that the Glud1-/- mouse also shows reduced GSIS (170), which supports the idea that Glud1 plays an *in vivo* role in glucose- as well as glutamine-stimulated insulin secretion. However, in spite of these extensive studies many questions remain regarding the mechanism linking Glud1 to insulin secretion.

In our experiments, overexpression of wild-type Glud1 failed to alter insulin secretion, while suppression of Glud1 reduced both AASIS and GSIS. As with the OGC and ICDc, it also appears that Glud1 is part of a common metabolic stimulatory pathway shared by multiple fuel secretagogues. There was no effect of Glud1 suppression on KCl-induced insulin secretion in the absence of metabolic stimulation (either low glucose or glutamine alone), suggesting that non-metabolic signaling pathways were unaffected. However, Glud1 suppression did reduce insulin secretion in the presence of KCl plus high glucose or glutamine and BCH, which indicated that metabolic signaling pathways were altered.

The decrease in AASIS after Glud1 suppression is most easily explained by reduced glutamate oxidation, as suggested by a previous study (153). This is further supported by the attenuated drop in glu* levels measured in our own experiments, and the lack of effect of Glud1 suppression on dm-2OG-stimulated secretion, which is presumed to deliver substrate for oxidation at a step downstream of the Glud1 reaction.

In contrast, the reduction in GSIS after Glud1 suppression was not due to changes in glucose usage or oxidation, and instead corresponded with decreased NADPH production, as observed in previous studies (109; 114).

These different mechanisms behind the effects on GSIS and AASIS, as well as the lack of effect of Glud1 suppression on dm-2OG-SIS, indicate that these three fuel secretagogues work through some metabolic pathways that are shared, and some that are divergent. One possibility is that glutamine-stimulated insulin secretion is more dependent on processes related to oxidation, whereas glucose-stimulated insulin secretion instead places a stronger requirement on the pyruvate cycling reactions. Dm-2OG-SIS may fall somewhere in between, requiring oxidation and transport through the OGC, but not flux through Glud1. It is important to note that most of the amino-acid experiments were performed with the goal of roughly matching stimulatory glutamine levels during AASIS with stimulatory glucose levels during GSIS, with no glucose present during AASIS. While this offered the best situation for observing impairment to secretion and making less-biased comparisons between differences in metabolism, it may not accurately reflect the *in vivo* environment that the β-cell experiences most frequently during stimulation.

Even so, a physiologic role for such differences between AASIS and GSIS still makes some sense, as removal of plasma glucose, and not amino acids, is the end goal of insulin secretion. Therefore, β-cells are likely most finely-tuned metabolically for glucose-sensing, with substrate flux through the pyruvate cycling reactions playing the major role in regulating secretion, while amino acids act in more of a supporting function by simply augmenting oxidation and anaplerosis after a meal, and not being the primary driving force behind these processes.

These results add to the evidence linking the pyruvate-isocitrate cycle and NADPH production with regulation of GSIS; however, it is important to recognize that a wide range of alternative processes have also been implicated in insulin secretion (including the production of glutamate, which was discussed in chapter 4). Therefore, it is likely that NADPH is not the universal coupling factor for all fuel substrates. Indeed, minimal increases in NADPH were observed after stimulation with glutamine plus BCH, while no changes in NADPH were measured after suppression of Glud1, the OGC, or ICDc, despite the fact that AASIS was substantially decreased. These data indicate that changes in alternative coupling factors and processes must be involved.

5.3 Additional processes/ mechanisms involved in glucose-sensing and ATP-independent insulin secretion

One of the biggest obstacles to identifying the second-messenger coupling factors required for ATP-independent insulin secretion is the fact that changes in a large and diverse number of processes are observed to occur upon stimulation of β-cells with fuel-secretagogues. Furthermore, it is highly likely that multiple events are necessary for sustained amplification of second-phase insulin secretion, which makes rescue of experiment-induced secretion deficits that much more difficult. I have previously discussed potential roles in insulin secretion in the β-cell for fatty acids, 2OG and α-ketoglutarate hydroxylases, glutamate and metabotropic glutamate receptors, glutamine, GTP, and NADPH. I wish to conclude by briefly covering several additional areas of β-cell stimulus-secretion coupling research that were not mentioned earlier.

Kv channels

NADPH, 2OG, and GTP can all be generated as by-products of the pyruvate-isocitrate cycling pathway, and multiple lines evidence have linked changes in NADPH levels with altered insulin secretion (23). As much of our work has focused on NADPH, it is important to discuss some of the potential molecular targets for NADPH, starting with the voltage-dependent K+ channels (Kv channels).

The Kv channels are responsible for the re-polarization of the β-cell by opening after initial depolarization and mediating outward rectifying K+ currents. The Kv2.1 is the major β-cell isoform (183), although multiple channels exist, with differences in voltage sensitivities and kinetics of activation and inactivation. Regulation of Kv channels has been shown to occur via α- or β-subunits, or by large N-terminal domains (184).

Based on the role of the Kv channels in the β-cell, a delay in opening of the channels during cell stimulation would be presumed to result in prolonged depolarization, with potential beneficial effects for GSIS. In support of this model is the finding that inhibition of Kv channels enhances GSIS (185). Furthermore, additional reports have suggested that the Kv channels are regulated by the redox state in the cell, while an increase in the ratio of cytosolic NADPH:NADP+ in patch-clamped β-cells was associated with an increased rate of inactivation of the Kv channel (186).

Alternatively, Kv channels may instead be regulated by glucose-induced activation of the group VIA phospholipase A2 (iPLA2beta) enzyme, which hydrolyzes membrane phospholipids and leads to accumulation of arachadonic acid (187); in support of this idea is the fact that glucose and carbachol fail to significantly inactivate Kv2.1 channels from iPLA2β-KO mice (188).

Recently, we have observed that both ICDc and CIC suppression leads to consistent downregulation of expression of a different Kv channel, Kv2.2. However, the precise role that changes in expression of this channel play in determining the effects of ICDc or CIC suppression on insulin secretion remains to be determined.

Together, these observations suggest a potentially important role for the Kv channels in β-cell function, which may or may not include regulation by fuel-stimulated NADPH-production.

Glutathione and redox sensing

Another potential role for NADPH in the β-cell involves regulation of the exocytotic machinery in the cell. Increased NADPH production would potentially change the redox state of glutathione through the activity of NADPH-dependent glutathione reductase. In its reduced form, glutathione could then be used to generate reduced glutaredoxin-1 (GRX-1), which in turn can modify proteins involved in exocytosis, such as t-SNARE proteins (125).

Addition of both GRX and NADPH to the interior of patch-clamped β-cells has previously been shown to potentiate exocytotic activity (115). Overexpression of Grx-1 was recently observed to increase insulin secretion in 832/13 cells by ~40%; additionally, suppression of GRX-1 decreased secretion, while suppression of thioredoxin-1 (TRX-1), a related protein, showed no effects on secretion (189).

Interestingly, the OGC has been reported to function as a carrier for GSH (190); however, the expected direction is into (not out of) the mitochondria, as GSH is only produced in the cytosol.

NAADP and Ca^{2+} release

Beyond regulation of redox state, NADPH and NADP+ may also be converted into additional compounds with potential biological activity within the β-cell, such as NAADP. NAADP is the most potent universal calcium-mobilizing second messenger agent identified to date (191), and has virtually identical structure with NADP+, with the exception of the substitution of an –NH2 group with an –OH group (192). NAADP can be generated from NADP+, but only two enzymes are known to catalyze this reaction: CD38 (found in virtually all tissues), and the *Aplysia* homolog ADP-ribosyl cyclase (193). Interestingly, CD38 also catalyzes the cyclization of NAD to cADPR, which is another calcium-mobilizing agent (194).

Mitchell, et al, has reported that NAADP stimulation of ryanodine receptors on the surface of the acidic secretory vesicles within the β-cell is necessary for insulin secretion, and involves calcium release from these vesicles, not the endoplasmic reticulum (195). However, CD38 is expressed on the surface of cells, with its catalytic domain outside of the cell, which indicates potential involvement in autocrine or paracrine signaling (196), but not internal production of NAADP.

Therefore, while these observations are suggestive of another potentially important role for NADPH/ NADP+, it is still unclear how NAADP production is regulated within the cell in response to fuel secretagogues, and what physiologic role it may play in *in vivo* β-cell function.

Glud1 and GOT1 reactions

As mentioned previously, the amino acid aspartate is one of the few metabolites whose levels actually decrease during GSIS. This observation suggests a potential role of aspartate levels for determining how long insulin secretion can occur in the presence of fuel stimulation. Importantly, decreasing expression of the malate-aspartate shuttle Aralar1 has previously been shown to reduce GSIS, while overexpression of Aralar1 actually increased secretion and enhanced mitochondrial metabolism (197). A possible explanation is that disruption of aspartate transport limits flux through the aspartate-aminotransferase (GOT2) reaction, which catalyzes the conversion of aspartate and 2OG to oxaloacetate and glutamate.

Recent studies by our lab suggest involvement of GOT2 in GSIS. Because a product of the GOT2 reaction is glutamate, it is possible that GOT2 and Glud1 may function in a mini-cycle within the mitochondria, or at least may work in a complementary manner. Glud1 is known to be a sensor of leucine, whereas transamination (by GOT2, and other enzymes) may instead play a role in KIC-stimulated insulin secretion; in support of this idea is the observation that AOA, a universal inhibitor of transamination reactions, markedly decreased the effects of KIC on insulin secretion, but actually potentiated the effects of leucine (198).

Clearly, additional work is needed to understand how these enzymes couple metabolism of glutamate, aspartate, and 2OG with insulin secretion.

DAG and cAMP

Both carbachol and glucose have been shown to increase insulin release in the absence of an increase in [Ca^{2+}] (199). This pathway was blocked by reducing GTP levels (200), but physiological role for this pathway has not yet been shown.

DAG and cAMP are two well-studied second messengers that have been hypothesized to augment exocytosis in a manner that may be calcium-independent (56; 59; 60) and involve activation of PKA. Alternatively, cAMP has been shown to enhance Ca^{2+}-stimulated insulin release, with no effects in the absence of stimulated exocytosis (201), by raising the release probability of the immediately releasable granules and also increasing the rate at which the pool is refilled (202)

Two agonists that are able to change the rate-limiting step in second phase secretion, the conversion of readily releasable insulin granules to the state of immediate releasability, are GLP-1 and acetylcholine. Exposure of β-cells to GLP-1 increases production of cAMP, while acetylcholine acts via production of DAG. However, the actual number of insulin granules released under either condition is still dependent and sensitive to changes in glucose concentration (48), indicating that DAG and cAMP production are not the only important factors.

Cytoskeleton rearrangement, vesicle trafficking and exocytosis

All cells undergo continuous exocytosis to some degree, but endocrine cells and neurons are capable of regulated exocytosis, which is characterized by the stable accumulation of cellular materials prior to release (203). Within the β-cell, insulin granules are known to exist in various functional pools (204) that undergo extensive movement (205) prior to secretion. Sustained insulin release is therefore dependent on glucose-derived signals that amplify and maintain secretion by facilitating vesicle mobilization and priming (52; 152).

Granule movement can be stimulated by glucose metabolism (206), ATP (207), or cAMP (208) and is important for second phase insulin secretion (209). ATP is essential for granule priming prior to exocytosis (210), and may potentially function through the inhibition of AMP kinase, which is thought to be a negative regulator of exocytosis. Inhibitors of AMP kinase enhance insulin secretion (211), while overexpression of a constitutively active mutant decreased glucose-stimulated granule movement (212). However, additional chemical modifications to the insulin-containing granules are necessary for exocytosis as well, including intra-vesicular acidification by a V-type H+ ATPase (213), and inhibition of acidification has been shown to prevent subsequent exocytosis (214).

Vesicle trafficking occurs along the microtubule network (215), and involves the microtubule-dependent ATPases kinesins (207; 216). Additionally, activation of small GTPases, such as Cdc42 (217), are also required for the second phase of insulin release (218). Glucose induces F-actin remodeling and rearrangement of the actin cytoskeleton (219), likely through inhibition of Cdc42 (219). In contrast, non-nutrient secretagogues are incapable of stimulating actin reorganization (220; 221).

Ultimately, once docked and primed, fusion of the insulin granules with the plasma membrane is accomplished with the help of the SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) (222), which act as calcium-sensors in exocytosis (223) and have been shown to interact with the voltage-dependent calcium channel (VDCC) (224).

Obviously, while much has been accomplished linking the events of fuel metabolism to in secretion, additional work is needed to identify the aspects of exocytosis most tightly-regulated by these processes as well as the metabolically-generated coupling factors involved.

5.4 Summary

The work presented in this dissertation expands upon previous studies investigating the role of metabolism in fuel-stimulated insulin secretion from pancreatic β-cells. Here, both glucose and glutamine were observed to require several common metabolic reactions for stimulating insulin release, involving flux through the mitochondrial inner-membrane 2-oxoglutarate carrier, the mitochondrial enzyme glutamate dehydrogenase, and the cytosolic NADP-dependent enzyme isocitrate dehydrogenase. These data indicate a shared metabolic pathway necessary for fuel-stimulated insulin release, and suggest that future insulin secretion studies would benefit from simultaneous investigation of both GSIS and AASIS.

While changes in metabolite levels, including glutamate and NADPH, were observed to correlate with altered GSIS, these metabolites were not well correlated with AASIS. The complexity of cellular metabolism confounds the ability of single experiments to sort out cause from effect, and requires the use of multiple approaches for stringent hypothesis testing. Similarly, the likely existence of several, if not many processes necessary for the full insulin-secretion response from the β-cell means that unrelated effects on metabolism may produce identical insulin release phenotypes, making the identification of the key required events that much more challenging.

However, in light of these difficulties, excellent progress has been made over the past several decades in understanding many of the events responsible stimulus-secretion coupling, and the ways in which these processes become dysregulated in disease states. The use of computer modeling will likely be necessary in future investigations of understanding metabolism (225; 226), and will only further help to elucidate the nuances of biochemical pathway flux in the β-cell.

References

1. Tirosh A, Shai I, Tekes-Manova D, Israeli E, Pereg D, Shochat T, Kochba I, Rudich A: Normal fasting plasma glucose levels and type 2 diabetes in young men. *N Engl J Med* 353:1454-1462, 2005

2. Clarke DD, Sokoloff L: Circulation and Energy Metabolism of the Brain. In *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects,* 5th ed. Siegal G, Agranoff B, Albers RW, Molinoff P, Eds. New York, New York, USA, Raven Press, 1994, p. 645-680

3. Rosenthal JM, Amiel SA, Yaguez L, Bullmore E, Hopkins D, Evans M, Pernet A, Reid H, Giampietro V, Andrew CM, Suckling J, Simmons A, Williams SC: The effect of acute hypoglycemia on brain function and activation: a functional magnetic resonance imaging study. *Diabetes* 50:1618-1626, 2001

4. Cryer PE: Glucose Homeostasis and Hypoglycemia. In *Williams Textbook of Endocrinology,* 10th ed. Larsen PR, Kronenberg H, Melmed S, Polonsky K, Eds. Philadelphia, Pennsylvania, USA, Saunders/Elsevier, 2003, p. 1585-1618

5. Cryer PE: Hypoglycemia, functional brain failure, and brain death. *J Clin Invest* 117:868-870, 2007

6. Langerhans P: Beitrage Zur Mikroscopischen Anatomie De Bauchspeicheldrusse. Berlin, Germany, 1869

7. Banting FG, Best CH, Macleod JJR, Noble EC: The effect of pancreatic extract (Insulin) on normal rabbits. *American Journal of Physiology* 62:162-176, 1922

8. Banting FG, Best CH, Macleod JJR, Noble EC: The effects of insulin on experimental hyperglycemia in rabbits. *American Journal of Physiology* 62:559-580, 1922

9. Ricordi C, Gray DW, Hering BJ, Kaufman DB, Warnock GL, Kneteman NM, Lake SP, London NJ, Socci C, Alejandro R, et al.: Islet isolation assessment in man and large animals. *Acta Diabetol Lat* 27:185-195, 1990

10. Orci L: The insulin factory: a tour of the plant surroundings and a visit to the assembly line. The Minkowski lecture 1973 revisited. *Diabetologia* 28:528-546, 1985

11. Goldsmith PC, Rose JC, Arimura A, Ganong WF: Ultrastructural localization of somatostatin in pancreatic islets of the rat. *Endocrinology* 97:1061-1064, 1975

12. Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L: Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc Natl Acad Sci U S A* 101:2924-2929, 2004

13. Kimmel JR, Hayden LJ, Pollock HG: Isolation and characterization of a new pancreatic polypeptide hormone. *J Biol Chem* 250:9369-9376, 1975

14. Baum J, Simons BE, Jr., Unger RH, Madison LL: Localization of glucagon in the alpha cells in the pancreatic islet by immunofluorescent technics. *Diabetes* 11:371-374, 1962

15. Halban PA: Cellular sources of new pancreatic beta cells and therapeutic implications for regenerative medicine. *Nat Cell Biol* 6:1021-1025, 2004

16. Genuth SM: The Endocrine System. In *Physiology,* 4th ed. Berne RM, Levy MN, Eds. St. Louis, MO, USA, Mosby, 1998, p. 779-1013

17. Malecki MT, Mlynarski W: Monogenic diabetes: implications for therapy of rare types of disease. *Diabetes Obes Metab* 10:607-616, 2008

18. Kim C, Newton KM, Knopp RH: Gestational diabetes and the incidence of type 2 diabetes: a systematic review. *Diabetes Care* 25:1862-1868, 2002

19. Shah SH, Hauser ER, Bain JR, Muehlbauer MJ, Haynes C, Stevens RD, Wenner BR, Dowdy ZE, Granger CB, Ginsburg GS, Newgard CB, Kraus WE: High heritability of metabolomic profiles in families burdened with premature cardiovascular disease. *Mol Syst Biol* 5:258, 2009

20. Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM: Prevalence of overweight and obesity in the United States, 1999-2004. *JAMA* 295:1549-1555, 2006

21. Chan JM, Rimm EB, Colditz GA, Stampfer MJ, Willett WC: Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. *Diabetes Care* 17:961-969, 1994

22. Pfeifer MA, Halter JB, Porte D, Jr.: Insulin secretion in diabetes mellitus. *Am J Med* 70:579-588, 1981

23. Jensen MV, Joseph JW, Ronnebaum SM, Burgess SC, Sherry AD, Newgard CB: Metabolic cycling in control of glucose-stimulated insulin secretion. *Am J Physiol Endocrinol Metab* 295:E1287-1297, 2008

24. Dean PM, Matthews EK: Electrical activity in pancreatic islet cells. *Nature* 219:389-390, 1968

25. Newgard CB, McGarry JD: Metabolic coupling factors in pancreatic beta-cell signal transduction. *Annu Rev Biochem* 64:689-719, 1995

26. Parrilla R, Gomez-Acebo J: Effect of 2-deoxyglucose on insulin secretion in the rabbit. *Horm Res* 4:331-339, 1973

27. Pozza G, Galansino G, Hoffeld H, Foa PP: Stimulation of insulin output by monosaccharides and monosaccharide derivatives. *Am J Physiol* 192:497-500, 1958

28. Orci L, Ravazzola M, Baetens D, Inman L, Amherdt M, Peterson RG, Newgard CB, Johnson JH, Unger RH: Evidence that down-regulation of beta-cell glucose transporters in non-insulin-dependent diabetes may be the cause of diabetic hyperglycemia. *Proc Natl Acad Sci U S A* 87:9953-9957, 1990

29. Becker TC, Noel RJ, Johnson JH, Lynch RM, Hirose H, Tokuyama Y, Bell GI, Newgard CB: Differential effects of overexpressed glucokinase and hexokinase I in isolated islets. Evidence for functional segregation of the high and low Km enzymes. *J Biol Chem* 271:390-394, 1996

30. MacDonald MJ: Estimates of glycolysis, pyruvate (de)carboxylation, pentose phosphate pathway, and methyl succinate metabolism in incapacitated pancreatic islets. *Arch Biochem Biophys* 305:205-214, 1993

31. Boyd AE, 3rd, Aguilar-Bryan L, Bryan J, Kunze DL, Moss L, Nelson DA, Rajan AS, Raef H, Xiang HD, Yaney GC: Sulfonylurea signal transduction. *Recent Prog Horm Res* 47:299-316; discussion 316-297, 1991

32. Bennett MK, Scheller RH: The molecular machinery for secretion is conserved from yeast to neurons. *Proc Natl Acad Sci U S A* 90:2559-2563, 1993

33. Keahey HH, Rajan AS, Boyd AE, 3rd, Kunze DL: Characterization of voltage-dependent Ca2+ channels in beta-cell line. *Diabetes* 38:188-193, 1989

34. Nelson TY, Gaines KL, Rajan AS, Berg M, Boyd AE, 3rd: Increased cytosolic calcium. A signal for sulfonylurea-stimulated insulin release from beta cells. *J Biol Chem* 262:2608-2612, 1987

35. Jacobsson G, Bean AJ, Scheller RH, Juntti-Berggren L, Deeney JT, Berggren PO, Meister B: Identification of synaptic proteins and their isoform mRNAs in compartments of pancreatic endocrine cells. *Proc Natl Acad Sci U S A* 91:12487-12491, 1994

36. Wiser O, Trus M, Hernandez A, Renstrom E, Barg S, Rorsman P, Atlas D: The voltage sensitive Lc-type Ca2+ channel is functionally coupled to the exocytotic machinery. *Proc Natl Acad Sci U S A* 96:248-253, 1999

37. Tsuboi T, Rutter GA: Insulin secretion by 'kiss-and-run' exocytosis in clonal pancreatic islet beta-cells. *Biochem Soc Trans* 31:833-836, 2003

38. Bryan J, Crane A, Vila-Carriles WH, Babenko AP, Aguilar-Bryan L: Insulin secretagogues, sulfonylurea receptors and K(ATP) channels. *Curr Pharm Des* 11:2699-2716, 2005

39. Cook DL, Hales CN: Intracellular ATP directly blocks K+ channels in pancreatic B-cells. *Nature* 311:271-273, 1984

40. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JPt, Boyd AE, 3rd, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA: Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* 268:423-426, 1995

41. Thomas PM, Cote GJ, Wohllk N, Haddad B, Mathew PM, Rabl W, Aguilar-Bryan L, Gagel RF, Bryan J: Mutations in the sulfonylurea receptor gene in familial persistent hyperinsulinemic hypoglycemia of infancy. *Science* 268:426-429, 1995

42. Remedi MS, Rocheleau JV, Tong A, Patton BL, McDaniel ML, Piston DW, Koster JC, Nichols CG: Hyperinsulinism in mice with heterozygous loss of K(ATP) channels. *Diabetologia* 49:2368-2378, 2006

43. Rorsman P, Trube G: Calcium and delayed potassium currents in mouse pancreatic beta-cells under voltage-clamp conditions. *J Physiol* 374:531-550, 1986

44. Shiota C, Larsson O, Shelton KD, Shiota M, Efanov AM, Hoy M, Lindner J, Kooptiwut S, Juntti-Berggren L, Gromada J, Berggren PO, Magnuson MA: Sulfonylurea receptor type 1 knock-out mice have intact feeding-stimulated insulin secretion despite marked impairment in their response to glucose. *J Biol Chem* 277:37176-37183, 2002

45. Nenquin M, Szollosi A, Aguilar-Bryan L, Bryan J, Henquin JC: Both triggering and amplifying pathways contribute to fuel-induced insulin secretion in the absence of sulfonylurea receptor-1 in pancreatic beta-cells. *J Biol Chem* 279:32316-32324, 2004

46. Szollosi A, Nenquin M, Aguilar-Bryan L, Bryan J, Henquin JC: Glucose stimulates Ca2+ influx and insulin secretion in 2-week-old beta-cells lacking ATP-sensitive K+ channels. *J Biol Chem* 282:1747-1756, 2007

47. Curry DL, Bennett LL, Grodsky GM: Dynamics of insulin secretion by the perfused rat pancreas. *Endocrinology* 83:572-584, 1968

48. Straub SG, Sharp GW: Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes Metab Res Rev* 18:451-463, 2002

49. Ashcroft FM, Harrison DE, Ashcroft SJ: Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells. *Nature* 312:446-448, 1984

50. Rhodes CJ, Halban PA: Newly synthesized proinsulin/insulin and stored insulin are released from pancreatic B cells predominantly via a regulated, rather than a constitutive, pathway. *J Cell Biol* 105:145-153, 1987

51. Barg S, Eliasson L, Renstrom E, Rorsman P: A subset of 50 secretory granules in close contact with L-type Ca2+ channels accounts for first-phase insulin secretion in mouse beta-cells. *Diabetes* 51 Suppl 1:S74-82, 2002

52. Gembal M, Gilon P, Henquin JC: 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, 1992

53. Komatsu M, Yajima H, Yamada S, Kaneko T, Sato Y, Yamauchi K, Hashizume K, Aizawa T: Augmentation of Ca2+-stimulated insulin release by glucose and long-chain fatty acids in rat pancreatic islets: free fatty acids mimic ATP-sensitive K+ channel-independent insulinotropic action of glucose. *Diabetes* 48:1543-1549, 1999

54. Henquin JC, Ravier MA, Nenquin M, Jonas JC, Gilon P: Hierarchy of the beta-cell signals controlling insulin secretion. *Eur J Clin Invest* 33:742-750, 2003

55. Panten U, Schwanstecher M, Wallasch A, Lenzen S: Glucose both inhibits and stimulates insulin secretion from isolated pancreatic islets exposed to maximally effective concentrations of sulfonylureas. *Naunyn Schmiedebergs Arch Pharmacol* 338:459-462, 1988

56. Komatsu M, Schermerhorn T, Aizawa T, Sharp GW: Glucose stimulation of insulin release in the absence of extracellular Ca2+ and in the absence of any increase in intracellular Ca2+ in rat pancreatic islets. *Proc Natl Acad Sci U S A* 92:10728-10732, 1995

57. Newgard CB, Matschinski FM: Substrate Control of Insulin Release. In *Handbook of Physiology* Jefferson AC, Ed. Oxford, UK, Oxford University Press, 2001, p. 125-152

58. Meglasson MD, Matschinsky FM: Pancreatic islet glucose metabolism and regulation of insulin secretion. *Diabetes Metab Rev* 2:163-214, 1986

59. Dyachok O, Idevall-Hagren O, Sagetorp J, Tian G, Wuttke A, Arrieumerlou C, Akusjarvi G, Gylfe E, Tengholm A: Glucose-induced cyclic AMP oscillations regulate pulsatile insulin secretion. *Cell Metab* 8:26-37, 2008

60. Walz HA, Wierup N, Vikman J, Manganiello VC, Degerman E, Eliasson L, Holst LS: Beta-cell PDE3B regulates Ca2+-stimulated exocytosis of insulin. *Cell Signal* 19:1505-1513, 2007

61. Pi J, Bai Y, Zhang Q, Wong V, Floering LM, Daniel K, Reece JM, Deeney JT, Andersen ME, Corkey BE, Collins S: Reactive oxygen species as a signal in glucose-stimulated insulin secretion. *Diabetes* 56:1783-1791, 2007

62. Leloup C, Tourrel-Cuzin C, Magnan C, Karaca M, Castel J, Carneiro L, Colombani AL, Ktorza A, Casteilla L, Penicaud L: Mitochondrial reactive oxygen species are obligatory signals for glucose-induced insulin secretion. *Diabetes* 58:673-681, 2009

63. Fahien LA, MacDonald MJ: The succinate mechanism of insulin release. *Diabetes* 51:2669-2676, 2002

64. Kibbey RG, Pongratz RL, Romanelli AJ, Wollheim CB, Cline GW, Shulman GI: Mitochondrial GTP regulates glucose-stimulated insulin secretion. *Cell Metab* 5:253-264, 2007

65. Prentki M, Vischer S, Glennon MC, Regazzi R, Deeney JT, Corkey BE: Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. *J Biol Chem* 267:5802-5810, 1992

66. Deeney JT, Gromada J, Hoy M, Olsen HL, Rhodes CJ, Prentki M, Berggren PO, Corkey BE: Acute stimulation with long chain acyl-CoA enhances exocytosis in insulin-secreting cells (HIT T-15 and NMRI beta-cells). *J Biol Chem* 275:9363-9368, 2000

67. Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR, Jr., Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, Muir AI: The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. *J Biol Chem* 278:11303-11311, 2003

68. Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, Tanaka H, Maruyama M, Satoh R, Okubo S, Kizawa H,

Komatsu H, Matsumura F, Noguchi Y, Shinohara T, Hinuma S, Fujisawa Y, Fujino M: Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* 422:173-176, 2003

69. Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H: The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. *Cell Metab* 1:245-258, 2005

70. Latour MG, Alquier T, Oseid E, Tremblay C, Jetton TL, Luo J, Lin DC, Poitout V: GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo. *Diabetes* 56:1087-1094, 2007

71. Nagasumi K, Esaki R, Iwachidow K, Yasuhara Y, Ogi K, Tanaka H, Nakata M, Yano T, Shimakawa K, Taketomi S, Takeuchi K, Odaka H, Kaisho Y: Overexpression of GPR40 in pancreatic beta-cells augments glucose-stimulated insulin secretion and improves glucose tolerance in normal and diabetic mice. *Diabetes* 58:1067-1076, 2009

72. Winzell MS, Ahren B: G-protein-coupled receptors and islet function-implications for treatment of type 2 diabetes. *Pharmacol Ther* 116:437-448, 2007

73. Antinozzi PA, Segall L, Prentki M, McGarry JD, Newgard CB: Molecular or pharmacologic perturbation of the link between glucose and lipid metabolism is without effect on glucose-stimulated insulin secretion. A re-evaluation of the long-chain acyl-CoA hypothesis. *J Biol Chem* 273:16146-16154, 1998

74. Mulder H, Lu D, Finley Jt, An J, Cohen J, Antinozzi PA, McGarry JD, Newgard CB: Overexpression of a modified human malonyl-CoA decarboxylase blocks the glucose-induced increase in malonyl-CoA level but has no impact on insulin secretion in INS-1-derived (832/13) beta-cells. *J Biol Chem* 276:6479-6484, 2001

75. Maechler P, Wollheim CB: Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* 402:685-689, 1999

76. Curtis DR, Phillis JW, Watkins JC: Chemical excitation of spinal neurones. *Nature* 183:611-612, 1959

77. Rubi B, Ishihara H, Hegardt FG, Wollheim CB, Maechler P: GAD65-mediated glutamate decarboxylation reduces glucose-stimulated insulin secretion in pancreatic beta cells. *J Biol Chem* 276:36391-36396, 2001

78. Li C, Buettger C, Kwagh J, Matter A, Daikhin Y, Nissim IB, Collins HW, Yudkoff M, Stanley CA, Matschinsky FM: A signaling role of glutamine in insulin secretion. *J Biol Chem* 279:13393-13401, 2004

79. Bertrand G, Ishiyama N, Nenquin M, Ravier MA, Henquin JC: The elevation of glutamate content and the amplification of insulin secretion in glucose-stimulated pancreatic islets are not causally related. *J Biol Chem* 277:32883-32891, 2002

80. Yamada S, Komatsu M, Sato Y, Yamauchi K, Aizawa T, Hashizume K: Glutamate is not a major conveyer of ATP-sensitive K+ channel-independent glucose action in pancreatic islet beta cell. *Endocr J* 48:391-395, 2001

81. Storto M, Capobianco L, Battaglia G, Molinaro G, Gradini R, Riozzi B, Di Mambro A, Mitchell KJ, Bruno V, Vairetti MP, Rutter GA, Nicoletti F: Insulin secretion is controlled by mGlu5 metabotropic glutamate receptors. *Mol Pharmacol* 69:1234-1241, 2006

82. Khan A, Ling ZC, Landau BR: Quantifying the carboxylation of pyruvate in pancreatic islets. *J Biol Chem* 271:2539-2542, 1996

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

84. MacDonald MJ, McKenzie DI, Walker TM, Kaysen JH: Lack of glyconeogenesis in pancreatic islets: expression of gluconeogenic enzyme genes in islets. *Horm Metab Res* 24:158-160, 1992

85. Brun T, Roche E, Assimacopoulos-Jeannet F, Corkey BE, Kim KH, Prentki M: Evidence for an anaplerotic/malonyl-CoA pathway in pancreatic beta-cell nutrient signaling. *Diabetes* 45:190-198, 1996

86. Cline GW, Lepine RL, Papas KK, Kibbey RG, Shulman GI: 13C NMR isotopomer analysis of anaplerotic pathways in INS-1 cells. *J Biol Chem* 279:44370-44375, 2004

87. Jeffrey FM, Storey CJ, Sherry AD, Malloy CR: 13C isotopomer model for estimation of anaplerotic substrate oxidation via acetyl-CoA. *Am J Physiol* 271:E788-799, 1996

88. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167-178, 1992

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

90. Lu D, Mulder H, Zhao P, Burgess SC, Jensen MV, Kamzolova S, Newgard CB, Sherry AD: 13C NMR isotopomer analysis reveals a connection between pyruvate cycling and glucose-stimulated insulin secretion (GSIS). *Proc Natl Acad Sci U S A* 99:2708-2713, 2002

91. Nicholls LI, Ainscow EK, Rutter GA: Glucose-stimulated insulin secretion does not require activation of pyruvate dehydrogenase: impact of adenovirus-mediated overexpression of PDH kinase and PDH phosphate phosphatase in pancreatic islets. *Biochem Biophys Res Commun* 291:1081-1088, 2002

92. Jensen MV, Joseph JW, Ilkayeva O, Burgess S, Lu D, Ronnebaum SM, Odegaard M, Becker TC, Sherry AD, Newgard CB: Compensatory responses to pyruvate carboxylase suppression in islet beta-cells. Preservation of glucose-stimulated insulin secretion. *J Biol Chem* 281:22342-22351, 2006

93. Bahl JJ, Matsuda M, DeFronzo RA, Bressler R: In vitro and in vivo suppression of gluconeogenesis by inhibition of pyruvate carboxylase. *Biochem Pharmacol* 53:67-74, 1997

94. Farfari S, Schulz V, Corkey B, Prentki M: Glucose-regulated anaplerosis and cataplerosis in pancreatic beta-cells: possible implication of a pyruvate/citrate shuttle in insulin secretion. *Diabetes* 49:718-726, 2000

95. Fransson U, Rosengren AH, Schuit FC, Renstrom E, Mulder H: Anaplerosis via pyruvate carboxylase is required for the fuel-induced rise in the ATP:ADP ratio in rat pancreatic islets. *Diabetologia* 49:1578-1586, 2006

96. Assimacopoulos-Jeannet F, Thumelin S, Roche E, Esser V, McGarry JD, Prentki M: Fatty acids rapidly induce the carnitine palmitoyltransferase I gene in the pancreatic beta-cell line INS-1. *J Biol Chem* 272:1659-1664, 1997

97. Randle PJ, Garland PB, Hales CN, Newsholme EA: The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1:785-789, 1963

98. Boucher A, Lu D, Burgess SC, Telemaque-Potts S, Jensen MV, Mulder H, Wang MY, Unger RH, Sherry AD, Newgard CB: Biochemical mechanism of lipid-induced impairment of glucose-stimulated insulin secretion and reversal with a malate analogue. *J Biol Chem* 279:27263-27271, 2004

99. Bollheimer LC, Skelly RH, Chester MW, McGarry JD, Rhodes CJ: Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. *J Clin Invest* 101:1094-1101, 1998

100. Liu YQ, Moibi JA, Leahy JL: Chronic high glucose lowers pyruvate dehydrogenase activity in islets through enhanced production of long chain acyl-CoA: prevention of impaired glucose oxidation by enhanced pyruvate recycling through the malate-pyruvate shuttle. *J Biol Chem* 279:7470-7475, 2004

101. Liu YQ, Jetton TL, Leahy JL: beta-Cell adaptation to insulin resistance. Increased pyruvate carboxylase and malate-pyruvate shuttle activity in islets of nondiabetic Zucker fatty rats. *J Biol Chem* 277:39163-39168, 2002

102. Macdonald MJ: Export of metabolites from pancreatic islet mitochondria as a means to study anaplerosis in insulin secretion. *Metabolism* 52:993-998, 2003

103. Heart E, Cline GW, Collis LP, Pongratz RL, Gray J, Smith PJ: Role for malic enzyme, pyruvate carboxylation and mitochondrial malate import in the glucose-stimulated insulin secretion. *Am J Physiol Endocrinol Metab*, 2009

104. Pongratz RL, Kibbey RG, Shulman GI, Cline GW: Cytosolic and mitochondrial malic enzyme isoforms differentially control insulin secretion. *J Biol Chem* 282:200-207, 2007

105. Ronnebaum SM, Jensen MV, Hohmeier HE, Burgess SC, Zhou YP, Qian S, MacNeil D, Howard A, Thornberry N, Ilkayeva O, Lu D, Sherry AD, Newgard CB: 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, 2008

106. Pongratz RL, Kibbey RG, Cline GW: Investigating the roles of mitochondrial and cytosolic malic enzyme in insulin secretion. *Methods Enzymol* 457:425-450, 2009

107. Teller JK, Fahien LA, Davis JW: Kinetics and regulation of hepatoma mitochondrial NAD(P) malic enzyme. *J Biol Chem* 267:10423-10432, 1992

108. Palmieri F: The mitochondrial transporter family (SLC25): physiological and pathological implications. *Pflugers Arch* 447:689-709, 2004

109. Joseph JW, Jensen MV, Ilkayeva O, Palmieri F, Alarcon C, Rhodes CJ, Newgard CB: The mitochondrial citrate/isocitrate carrier plays a regulatory role in glucose-stimulated insulin secretion. *J Biol Chem* 281:35624-35632, 2006

110. Kaplan RS: Structure and function of mitochondrial anion transport proteins. *J Membr Biol* 179:165-183, 2001

111. Joseph JW, Odegaard ML, Ronnebaum SM, Burgess SC, Muehlbauer J, Sherry AD, Newgard CB: Normal flux through ATP-citrate lyase or fatty acid synthase is not required for glucose-stimulated insulin secretion. *J Biol Chem* 282:31592-31600, 2007

112. Ronnebaum SM, Joseph JW, Ilkayeva O, Burgess SC, Lu D, Becker TC, Sherry AD, Newgard CB: Chronic suppression of acetyl CoA carboxylase 1 in beta cells impairs insulin secretion via inhibition of glucose rather than lipid metabolism. *J Biol Chem*, 2008

113. Chakravarthy MV, Zhu Y, Lopez M, Yin L, Wozniak DF, Coleman T, Hu Z, Wolfgang M, Vidal-Puig A, Lane MD, Semenkovich CF: Brain fatty acid synthase activates PPARalpha to maintain energy homeostasis. *J Clin Invest* 117:2539-2552, 2007

114. Ronnebaum SM, Ilkayeva O, Burgess SC, Joseph JW, Lu D, Stevens RD, Becker TC, Sherry AD, Newgard CB, Jensen MV: A pyruvate cycling pathway involving cytosolic NADP-dependent isocitrate dehydrogenase regulates glucose-stimulated insulin secretion. *J Biol Chem* 281:30593-30602, 2006

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

116. Rabaglia ME, Gray-Keller MP, Frey BL, Shortreed MR, Smith LM, Attie AD: Alpha-Ketoisocaproate-induced hypersecretion of insulin by islets from diabetes-susceptible mice. *Am J Physiol Endocrinol Metab* 289:E218-224, 2005

117. Palmisano A, Zara V, Honlinger A, Vozza A, Dekker PJ, Pfanner N, Palmieri F: Targeting and assembly of the oxoglutarate carrier: general principles for biogenesis of carrier proteins of the mitochondrial inner membrane. *Biochem J* 333 (Pt 1):151-158, 1998

118. Schatz G, Dobberstein B: Common principles of protein translocation across membranes. *Science* 271:1519-1526, 1996

119. Neupert W: Protein import into mitochondria. *Annu Rev Biochem* 66:863-917, 1997

120. Koehler CM, Jarosch E, Tokatlidis K, Schmid K, Schweyen RJ, Schatz G: Import of mitochondrial carriers mediated by essential proteins of the intermembrane space. *Science* 279:369-373, 1998

121. Hackenberg H, Klingenberg M: Molecular weight and hydrodynamic parameters of the adenosine 5'-diphosphate--adenosine 5'-triphosphate carrier in Triton X-100. *Biochemistry* 19:548-555, 1980

122. Brandolin G, Doussiere J, Gulik A, Gulik-Krzywicki T, Lauquin GJ, Vignais PV: Kinetic, binding and ultrastructural properties of the beef heart adenine nucleotide carrier protein after incorporation into phospholipid vesicles. *Biochim Biophys Acta* 592:592-614, 1980

123. Brandolin G, Le Saux A, Trezeguet V, Lauquin GJ, Vignais PV: Chemical, immunological, enzymatic, and genetic approaches to studying the arrangement of the peptide chain of the ADP/ATP carrier in the mitochondrial membrane. *J Bioenerg Biomembr* 25:459-472, 1993

124. Palmieri L, Agrimi G, Runswick MJ, Fearnley IM, Palmieri F, Walker JE: Identification in Saccharomyces cerevisiae of two isoforms of a novel mitochondrial transporter for 2-oxoadipate and 2-oxoglutarate. *J Biol Chem* 276:1916-1922, 2001

125. MacDonald MJ, Fahien LA, Brown LJ, Hasan NM, Buss JD, Kendrick MA: Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion. *Am J Physiol Endocrinol Metab* 288:E1-15, 2005

126. Weinhaus AJ, Poronnik P, Tuch BE, Cook DI: Mechanisms of arginine-induced increase in cytosolic calcium concentration in the beta-cell line NIT-1. *Diabetologia* 40:374-382, 1997

127. Kim W, Egan JM: The role of incretins in glucose homeostasis and diabetes treatment. *Pharmacol Rev* 60:470-512, 2008

128. Lewis RJ, Garcia ML: Therapeutic potential of venom peptides. *Nat Rev Drug Discov* 2:790-802, 2003

129. Chia CW, Egan JM: Incretin-based therapies in type 2 diabetes mellitus. *J Clin Endocrinol Metab* 93:3703-3716, 2008

130. MacDonald MJ: Elusive proximal signals of beta-cells for insulin secretion. *Diabetes* 39:1461-1466, 1990

131. Zhao C, Wilson MC, Schuit F, Halestrap AP, Rutter GA: Expression and distribution of lactate/monocarboxylate transporter isoforms in pancreatic islets and the exocrine pancreas. *Diabetes* 50:361-366, 2001

132. MacDonald MJ: Differences between mouse and rat pancreatic islets: succinate responsiveness, malic enzyme, and anaplerosis. *Am J Physiol Endocrinol Metab* 283:E302-310, 2002

133. Willenborg M, Panten U, Rustenbeck I: Triggering and amplification of insulin secretion by dimethyl alpha-ketoglutarate, a membrane permeable alpha-ketoglutarate analogue. *Eur J Pharmacol* 607:41-46, 2009

134. Arco AD, Satrustegui J: New mitochondrial carriers: an overview. *Cell Mol Life Sci* 62:2204-2227, 2005

135. Haigis MC, Mostoslavsky R, Haigis KM, Fahie K, Christodoulou DC, Murphy AJ, Valenzuela DM, Yancopoulos GD, Karow M, Blander G, Wolberger C, Prolla TA, Weindruch R, Alt FW, Guarente L: SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells. *Cell* 126:941-954, 2006

136. Smith TJ, Stanley CA: Untangling the glutamate dehydrogenase allosteric nightmare. *Trends Biochem Sci* 33:557-564, 2008

137. Stanley CA, Lieu YK, Hsu BY, Burlina AB, Greenberg CR, Hopwood NJ, Perlman K, Rich BH, Zammarchi E, Poncz M: Hyperinsulinism and hyperammonemia in infants with regulatory mutations of the glutamate dehydrogenase gene. *N Engl J Med* 338:1352-1357, 1998

138. Becker TC, Noel RJ, Coats WS, Gomez-Foix AM, Alam T, Gerard RD, Newgard CB: Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol* 43 Pt A:161-189, 1994

139. Schisler JC, Jensen PB, Taylor DG, Becker TC, Knop FK, Takekawa S, German M, Weir GC, Lu D, Mirmira RG, Newgard CB: The Nkx6.1 homeodomain transcription factor suppresses glucagon expression and regulates glucose-stimulated insulin secretion in islet beta cells. *Proc Natl Acad Sci U S A* 102:7297-7302, 2005

140. Bain JR, Schisler JC, Takeuchi K, Newgard CB, Becker TC: An adenovirus vector for efficient RNA interference-mediated suppression of target genes in insulinoma cells and pancreatic islets of langerhans. *Diabetes* 53:2190-2194, 2004

141. Naber SP, McDonald JM, Jarett L, McDaniel ML, Ludvigsen CW, Lacy PE: Preliminary characterization of calcium binding in islet-cell plasma membranes. *Diabetologia* 19:439-444, 1980

142. Milburn JL, Jr., Hirose H, Lee YH, Nagasawa Y, Ogawa A, Ohneda M, BeltrandelRio H, Newgard CB, Johnson JH, Unger RH: Pancreatic beta-cells in obesity. Evidence for induction of functional, morphologic, and metabolic abnormalities by increased long chain fatty acids. *J Biol Chem* 270:1295-1299, 1995

143. Joseph JW, Koshkin V, Saleh MC, Sivitz WI, Zhang CY, Lowell BB, Chan CB, Wheeler MB: Free fatty acid-induced beta-cell defects are dependent on uncoupling protein 2 expression. *J Biol Chem* 279:51049-51056, 2004

144. Kanao T, Kawamura M, Fukui T, Atomi H, Imanaka T: Characterization of isocitrate dehydrogenase from the green sulfur bacterium Chlorobium limicola. A carbon dioxide-fixing enzyme in the reductive tricarboxylic acid cycle. *Eur J Biochem* 269:1926-1931, 2002

145. Fujioka H, Okano Y, Inada H, Asada M, Kawamura T, Hase Y, Yamano T: Molecular characterisation of glutamate dehydrogenase gene defects in Japanese patients with congenital hyperinsulinism/hyperammonaemia. *Eur J Hum Genet* 9:931-937, 2001

146. Hughes SD, Quaade C, Johnson JH, Ferber S, Newgard CB: Transfection of AtT-20ins cells with GLUT-2 but not GLUT-1 confers glucose-stimulated insulin secretion. Relationship to glucose metabolism. *J Biol Chem* 268:15205-15212, 1993

147. Kim JY, Koves TR, Yu GS, Gulick T, Cortright RN, Dohm GL, Muoio DM: Evidence of a malonyl-CoA-insensitive carnitine palmitoyltransferase I activity in red skeletal muscle. *Am J Physiol Endocrinol Metab* 282:E1014-1022, 2002

148. Schultz V, Sussman I, Bokvist K, Tornheim K: Bioluminometric assay of ADP and ATP at high ATP/ADP ratios: assay of ADP after enzymatic removal of ATP. *Anal Biochem* 215:302-304, 1993

149. Passonneau JV, Lowry OH: Enzymatic Analysis: A Practical Guide. Totowna, NJ, Humana Press, Inc, 1993

150. An J, Muoio DM, Shiota M, Fujimoto Y, Cline GW, Shulman GI, Koves TR, Stevens R, Millington D, Newgard CB: Hepatic expression of malonyl-CoA decarboxylase reverses muscle, liver and whole-animal insulin resistance. *Nat Med* 10:268-274, 2004

151. Wu JY, Kao HJ, Li SC, Stevens R, Hillman S, Millington D, Chen YT: ENU mutagenesis identifies mice with mitochondrial branched-chain aminotransferase deficiency resembling human maple syrup urine disease. *J Clin Invest* 113:434-440, 2004

152. Henquin JC: Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 49:1751-1760, 2000

153. Li C, Allen A, Kwagh J, Doliba NM, Qin W, Najafi H, Collins HW, Matschinsky FM, Stanley CA, Smith TJ: Green tea polyphenols modulate insulin secretion by inhibiting glutamate dehydrogenase. *J Biol Chem* 281:10214-10221, 2006

154. Indiveri C, Palmieri F, Bisaccia F, Kramer R: Kinetics of the reconstituted 2-oxoglutarate carrier from bovine heart mitochondria. *Biochim Biophys Acta* 890:310-318, 1987

155. Palmieri F, Bisaccia F, Capobianco L, Dolce V, Fiermonte G, Iacobazzi V, Zara V: Transmembrane topology, genes, and biogenesis of the mitochondrial phosphate and oxoglutarate carriers. *J Bioenerg Biomembr* 25:493-501, 1993

156. Hasan NM, Longacre MJ, Stoker SW, Boonsaen T, Jitrapakdee S, Kendrick MA, Wallace JC, MacDonald MJ: Impaired anaplerosis and insulin secretion in insulinoma cells caused by small interfering RNA-mediated suppression of pyruvate carboxylase. *J Biol Chem* 283:28048-28059, 2008

157. Fiermonte G, Dolce V, Palmieri L, Ventura M, Runswick MJ, Palmieri F, Walker JE: Identification of the human mitochondrial oxodicarboxylate carrier. Bacterial expression, reconstitution, functional characterization, tissue distribution, and chromosomal location. *J Biol Chem* 276:8225-8230, 2001

158. Fernandez-Pascual S, Mukala-Nsengu-Tshibangu A, Martin Del Rio R, Tamarit-Rodriguez J: Conversion into GABA (gamma-aminobutyric acid) may reduce the capacity of L-glutamine as an insulin secretagogue. *Biochem J* 379:721-729, 2004

159. Malaisse-Lagae F, Welsh M, Lebrun P, Herchuelz A, Sener A, Hellerstrom C, Malaisse WJ: The stimulus-secretion coupling of amino acid-induced insulin release. Secretory and oxidative response of pancreatic islets to L-asparagine. *Diabetes* 33:464-469, 1984

160. Panten U, Rustenbeck I: Fuel-induced amplification of insulin secretion in mouse pancreatic islets exposed to a high sulfonylurea concentration: role of the NADPH/NADP+ ratio. *Diabetologia* 51:101-109, 2008

161. Ozer A, Bruick RK: Non-heme dioxygenases: cellular sensors and regulators jelly rolled into one? *Nat Chem Biol* 3:144-153, 2007

162. Hsu BY, Kelly A, Thornton PS, Greenberg CR, Dilling LA, Stanley CA: Protein-sensitive and fasting hypoglycemia in children with the hyperinsulinism/hyperammonemia syndrome. *J Pediatr* 138:383-389, 2001

163. MacMullen C, Fang J, Hsu BY, Kelly A, de Lonlay-Debeney P, Saudubray JM, Ganguly A, Smith TJ, Stanley CA: Hyperinsulinism/hyperammonemia syndrome in children with regulatory mutations in the inhibitory guanosine triphosphate-binding domain of glutamate dehydrogenase. *J Clin Endocrinol Metab* 86:1782-1787, 2001

164. Michaelidis TM, Tzimagiorgis G, Moschonas NK, Papamatheakis J: The human glutamate dehydrogenase gene family: gene organization and structural characterization. *Genomics* 16:150-160, 1993

165. Hudson RC, Daniel RM: L-glutamate dehydrogenases: distribution, properties and mechanism. *Comp Biochem Physiol B* 106:767-792, 1993

166. Fahien LA, MacDonald MJ, Kmiotek EH, Mertz RJ, Fahien CM: Regulation of insulin release by factors that also modify glutamate dehydrogenase. *J Biol Chem* 263:13610-13614, 1988

167. Sener A, Malaisse-Lagae F, Malaisse WJ: Stimulation of pancreatic islet metabolism and insulin release by a nonmetabolizable amino acid. *Proc Natl Acad Sci U S A* 78:5460-5464, 1981

168. Tanizawa Y, Nakai K, Sasaki T, Anno T, Ohta Y, Inoue H, Matsuo K, Koga M, Furukawa S, Oka Y: Unregulated elevation of glutamate dehydrogenase activity induces glutamine-stimulated insulin secretion: identification and characterization of a GLUD1 gene mutation and insulin secretion studies with MIN6 cells overexpressing the mutant glutamate dehydrogenase. *Diabetes* 51:712-717, 2002

169. Li C, Matter A, Kelly A, Petty TJ, Najafi H, MacMullen C, Daikhin Y, Nissim I, Lazarow A, Kwagh J, Collins HW, Hsu BY, Nissim I, Yudkoff M, Matschinsky FM, Stanley CA: Effects of a GTP-insensitive mutation of glutamate dehydrogenase on insulin secretion in transgenic mice. *J Biol Chem* 281:15064-15072, 2006

170. Carobbio S, Frigerio F, Rubi B, Vetterli L, Bloksgaard M, Gjinovci A, Pournourmohammadi S, Herrera PL, Reith W, Mandrup S, Maechler P: Deletion of glutamate dehydrogenase in beta-cells abolishes part of the insulin secretory response not required for glucose homeostasis. *J Biol Chem* 284:921-929, 2009

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

172. Shimizu M, Weinstein IB: Modulation of signal transduction by tea catechins and related phytochemicals. *Mutat Res* 591:147-160, 2005

173. Masuda M, Suzui M, Weinstein IB: Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression, and chemosensitivity in human head and neck squamous cell carcinoma cell lines. *Clin Cancer Res* 7:4220-4229, 2001

174. Punathil T, Tollefsbol TO, Katiyar SK: EGCG inhibits mammary cancer cell migration through inhibition of nitric oxide synthase and guanylate cyclase. *Biochem Biophys Res Commun* 375:162-167, 2008

175. Patra SK, Rizzi F, Silva A, Rugina DO, Bettuzzi S: Molecular targets of (-)-epigallocatechin-3-gallate (EGCG): specificity and interaction with membrane lipid rafts. *J Physiol Pharmacol* 59 Suppl 9:217-235, 2008

176. Maechler P, Antinozzi PA, Wollheim CB: Modulation of glutamate generation in mitochondria affects hormone secretion in INS-1E beta cells. *IUBMB Life* 50:27-31, 2000

177. Sener A, Conget I, Rasschaert J, Leclercq-Meyer V, Villanueva-Penacarrillo ML, Valverde I, Malaisse WJ: Insulinotropic action of glutamic acid dimethyl ester. *Am J Physiol* 267:E573-584, 1994

178. MacDonald MJ, Fahien LA: Glutamate is not a messenger in insulin secretion. *J Biol Chem* 275:34025-34027, 2000

179. Smith E, et al.: Glutamate Dehydrogenases. In *The Enzymes* Boyer P, Ed., Academic Press, 1975, p. 293-367

180. Brice NL, Varadi A, Ashcroft SJ, Molnar E: Metabotropic glutamate and GABA(B) receptors contribute to the modulation of glucose-stimulated insulin secretion in pancreatic beta cells. *Diabetologia* 45:242-252, 2002

181. Hayashi M, Yamada H, Uehara S, Morimoto R, Muroyama A, Yatsushiro S, Takeda J, Yamamoto A, Moriyama Y: Secretory granule-mediated co-secretion of L-glutamate and glucagon triggers glutamatergic signal transmission in islets of Langerhans. *J Biol Chem* 278:1966-1974, 2003

182. Brownlee M: The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54:1615-1625, 2005

183. MacDonald PE, Ha XF, Wang J, Smukler SR, Sun AM, Gaisano HY, Salapatek AM, Backx PH, Wheeler MB: Members of the Kv1 and Kv2 voltage-dependent K(+) channel families regulate insulin secretion. *Mol Endocrinol* 15:1423-1435, 2001

184. MacDonald PE, Joseph JW, Rorsman P: Glucose-sensing mechanisms in pancreatic beta-cells. *Philos Trans R Soc Lond B Biol Sci* 360:2211-2225, 2005

185. MacDonald PE, Sewing S, Wang J, Joseph JW, Smukler SR, Sakellaropoulos G, Saleh MC, Chan CB, Tsushima RG, Salapatek AM, Wheeler MB: Inhibition of Kv2.1 voltage-dependent K+ channels in pancreatic beta-cells enhances glucose-dependent insulin secretion. *J Biol Chem* 277:44938-44945, 2002

186. MacDonald PE, Salapatek AM, Wheeler MB: Temperature and redox state dependence of native Kv2.1 currents in rat pancreatic beta-cells. *J Physiol* 546:647-653, 2003

187. Jacobson DA, Philipson LH: Action potentials and insulin secretion: new insights into the role of Kv channels. *Diabetes Obes Metab* 9 Suppl 2:89-98, 2007

188. Jacobson DA, Weber CR, Bao S, Turk J, Philipson LH: Modulation of the pancreatic islet beta-cell-delayed rectifier potassium channel Kv2.1 by the polyunsaturated fatty acid arachidonate. *J Biol Chem* 282:7442-7449, 2007

189. Reinbothe TM, Ivarsson R, Li DQ, Niazi O, Jing X, Zhang E, Stenson L, Bryborn U, Renstrom E: Glutaredoxin-1 mediates NADPH-dependent stimulation of calcium-dependent insulin secretion. *Mol Endocrinol* 23:893-900, 2009

190. Lash LH: Mitochondrial glutathione transport: physiological, pathological and toxicological implications. *Chem Biol Interact* 163:54-67, 2006

191. Guse AH, Lee HC: NAADP: a universal Ca2+ trigger. *Sci Signal* 1:re10, 2008

192. Lee HC, Aarhus R: A derivative of NADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose. *J Biol Chem* 270:2152-2157, 1995

193. Aarhus R, Graeff RM, Dickey DM, Walseth TF, Lee HC: ADP-ribosyl cyclase and CD38 catalyze the synthesis of a calcium-mobilizing metabolite from NADP. *J Biol Chem* 270:30327-30333, 1995

194. Howard M, Grimaldi JC, Bazan JF, Lund FE, Santos-Argumedo L, Parkhouse RM, Walseth TF, Lee HC: Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science* 262:1056-1059, 1993

195. Mitchell KJ, Lai FA, Rutter GA: Ryanodine receptor type I and nicotinic acid adenine dinucleotide phosphate receptors mediate Ca2+ release from insulin-containing vesicles in living pancreatic beta-cells (MIN6). *J Biol Chem* 278:11057-11064, 2003

196. De Flora A, Zocchi E, Guida L, Franco L, Bruzzone S: Autocrine and paracrine calcium signaling by the CD38/NAD+/cyclic ADP-ribose system. *Ann N Y Acad Sci* 1028:176-191, 2004

197. Rubi B, del Arco A, Bartley C, Satrustegui J, Maechler P: 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, 2004

198. Gao Z, Young RA, Li G, Najafi H, Buettger C, Sukumvanich SS, Wong RK, Wolf BA, Matschinsky FM: Distinguishing features of leucine and alpha-ketoisocaproate sensing in pancreatic beta-cells. *Endocrinology* 144:1949-1957, 2003

199. Komatsu M, Schermerhorn T, Straub SG, Sharp GW: Pituitary adenylate cyclase-activating peptide, carbachol, and glucose stimulate insulin release in the absence of an increase in intracellular Ca2+. *Mol Pharmacol* 50:1047-1054, 1996

200. Komatsu M, Noda M, Sharp GW: Nutrient augmentation of Ca2+-dependent and Ca2+-independent pathways in stimulus-coupling to insulin secretion can be distinguished by their guanosine triphosphate requirements: studies on rat pancreatic islets. *Endocrinology* 139:1172-1183, 1998

201. Sharp GW: The adenylate cyclase-cyclic AMP system in islets of Langerhans and its role in the control of insulin release. *Diabetologia* 16:287-296, 1979

202. Renstrom E, Eliasson L, Rorsman P: Protein kinase A-dependent and -independent stimulation of exocytosis by cAMP in mouse pancreatic B-cells. *J Physiol* 502 (Pt 1):105-118, 1997

203. Gerber SH, Sudhof TC: Molecular determinants of regulated exocytosis. *Diabetes* 51 Suppl 1:S3-11, 2002

204. Rorsman P, Renstrom E: Insulin granule dynamics in pancreatic beta cells. *Diabetologia* 46:1029-1045, 2003

205. Somers G, Blondel B, Orci L, Malaisse WJ: Motile events in pancreatic endocrine cells. *Endocrinology* 104:255-264, 1979

206. Pouli AE, Emmanouilidou E, Zhao C, Wasmeier C, Hutton JC, Rutter GA: Secretory-granule dynamics visualized in vivo with a phogrin-green fluorescent protein chimaera. *Biochem J* 333 (Pt 1):193-199, 1998

207. Varadi A, Ainscow EK, Allan VJ, Rutter GA: Involvement of conventional kinesin in glucose-stimulated secretory granule movements and exocytosis in clonal pancreatic beta-cells. *J Cell Sci* 115:4177-4189, 2002

208. Hisatomi M, Hidaka H, Niki I: Ca2+/calmodulin and cyclic 3,5' adenosine monophosphate control movement of secretory granules through protein phosphorylation/dephosphorylation in the pancreatic beta-cell. *Endocrinology* 137:4644-4649, 1996

209. Farshori PQ, Goode D: Effects of the microtubule depolymerizing and stabilizing agents Nocodazole and taxol on glucose-induced insulin secretion from hamster islet tumor (HIT) cells. *J Submicrosc Cytol Pathol* 26:137-146, 1994

210. Eliasson L, Renstrom E, Ding WG, Proks P, Rorsman P: Rapid ATP-dependent priming of secretory granules precedes Ca(2+)-induced exocytosis in mouse pancreatic B-cells. *J Physiol* 503 (Pt 2):399-412, 1997

211. da Silva Xavier G, Leclerc I, Varadi A, Tsuboi T, Moule SK, Rutter GA: Role for AMP-activated protein kinase in glucose-stimulated insulin secretion and preproinsulin gene expression. *Biochem J* 371:761-774, 2003

212. Tsuboi T, da Silva Xavier G, Leclerc I, Rutter GA: 5'-AMP-activated protein kinase controls insulin-containing secretory vesicle dynamics. *J Biol Chem* 278:52042-52051, 2003

213. Renstrom E, Ivarsson R, Shears SB: Inositol 3,4,5,6-tetrakisphosphate inhibits insulin granule acidification and fusogenic potential. *J Biol Chem* 277:26717-26720, 2002

214. Barg S, Huang P, Eliasson L, Nelson DJ, Obermuller S, Rorsman P, Thevenod F, Renstrom E: Priming of insulin granules for exocytosis by granular Cl(-) uptake and acidification. *J Cell Sci* 114:2145-2154, 2001

215. Malaisse WJ, Van Obberghen E, Devis G, Somers G, Ravazzola M: Dynamics of insulin release and microtubular-microfilamentous system. V. A model for the phasic release of insulin. *Eur J Clin Invest* 4:313-318, 1974

216. Balczon R, Overstreet KA, Zinkowski RP, Haynes A, Appel M: The identification, purification, and characterization of a pancreatic beta-cell form of the microtubule adenosine triphosphatase kinesin. *Endocrinology* 131:331-336, 1992

217. Kowluru A, Seavey SE, Li G, Sorenson RL, Weinhaus AJ, Nesher R, Rabaglia ME, Vadakekalam J, Metz SA: Glucose- and GTP-dependent stimulation of the carboxyl methylation of CDC42 in rodent and human pancreatic islets and pure beta cells. Evidence for an essential role of GTP-binding proteins in nutrient-induced insulin secretion. *J Clin Invest* 98:540-555, 1996

218. Wang Z, Oh E, Thurmond DC: Glucose-stimulated Cdc42 signaling is essential for the second phase of insulin secretion. *J Biol Chem* 282:9536-9546, 2007

219. Nevins AK, Thurmond DC: Glucose regulates the cortical actin network through modulation of Cdc42 cycling to stimulate insulin secretion. *Am J Physiol Cell Physiol* 285:C698-710, 2003

220. Wilson JR, Ludowyke RI, Biden TJ: A redistribution of actin and myosin IIA accompanies Ca(2+)-dependent insulin secretion. *FEBS Lett* 492:101-106, 2001

221. Wang Z, Thurmond DC: Mechanisms of biphasic insulin-granule exocytosis - roles of the cytoskeleton, small GTPases and SNARE proteins. *J Cell Sci* 122:893-903, 2009

222. Chen YA, Scheller RH: SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol* 2:98-106, 2001

223. Meldolesi J, Chieregatti E: Fusion has found its calcium sensor. *Nat Cell Biol* 6:476-478, 2004

224. Catterall WA: Interactions of presynaptic Ca2+ channels and snare proteins in neurotransmitter release. *Ann N Y Acad Sci* 868:144-159, 1999

225. Mendes P, Hoops S, Sahle S, Gauges R, Dada J, Kummer U: Computational modeling of biochemical networks using COPASI. *Methods Mol Biol* 500:17-59, 2009

226. Hoops S, Sahle S, Gauges R, Lee C, Pahle J, Simus N, Singhal M, Xu L, Mendes P, Kummer U: COPASI--a COmplex PAthway SImulator. *Bioinformatics* 22:3067-3074, 2006

Biography

Born

July 26, 1980, St. Paul, MN

Education

Ph.D., Department of Pharmacology and Cancer Biology
Duke University, Durham, NC 2003-2009
Advisor: Dr. Chris Newgard

B.S. Biology, Minor Statistics
Iowa State University, Ames, IA 1999-2003

Peer-Reviewed Publications

Flux through the alpha-ketoglutarate/ 2-oxoglutarate carrier is required for fuel-stimulated insulin secretion. (in progress)
Odegaard ML, Joseph JW, Jensen MV, Ilkayeva O, Lu D, Newgard CB.

Glutamate dehydrogenase plays a critical role in both glucose- and glutamine-stimulated insulin secretion. (in progress)
Odegaard ML, Jensen MV, Lu D, Ilkayeva O, Ramsey C, Newgard CB.

Normal flux through ATP-citrate lyase or fatty acid synthase is not required for glucose-stimulated insulin secretion.
J Biol Chem. 2007 Oct 26; 282(43):31592-600.
Joseph JW, Odegaard ML, Ronnebaum SM, Burgess SC, Muehlbauer J, Sherry AD, Newgard CB.

Compensatory responses to pyruvate carboxylase suppression in islet beta-cells.
J Biol Chem. 2006 Aug 4; 281(31):22342-51.
Jensen MV, Joseph JW, Ilkayeva O, Burgess S, Lu D, Ronnebaum SM, Odegaard M, Becker TC, Sherry AD, Newgard CB.

Honors and Awards:

Duke University, Durham, NC

Recipient of the James B. Duke Scholarship

Society of Duke Fellows

Iowa State University, Ames, IA

Phi Beta Kappa Honor Society

Phi Kappa Phi Honor Society (top 5% LAS)