Stimulus/Secretion Coupling Factors in Glucose-Stimulated Insulin Secretion

Insights Gained From a Multidisciplinary Approach

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There is a growing appreciation for the complexity of the pathways involved in glucose-stimulated insulin secretion (GSIS) from pancreatic islet β-cells. In our laboratory, this has stimulated the development of an interdisciplinary approach to the problem. In this study, we review recent studies combining the tools of recombinant adenovirus for gene delivery, the development of novel cell lines that exhibit either robust or weak GSIS, and nuclear magnetic resonance imaging for metabolic fingerprinting of glucose-stimulated cells. Using these tools, we demonstrate a potentially important role for pyruvate carboxylase–mediated pyruvate cycling pathways in the control of GSIS, and discuss potential coupling factors produced by such pathways. *Diabetes* 51 (Suppl. 3):S389–S393, 2002

A fundamental property of pancreatic islet β-cells is their capacity to secrete insulin in response to changes in glucose concentrations. A complete understanding of the biochemical mechanism of glucose-stimulated insulin secretion (GSIS) would be extremely valuable for the development of new therapies for both major forms of diabetes. GSIS is perhaps the seminal example of metabolism as a signaling mechanism. However, unlike classic hormonal signaling pathways involving well-characterized receptors and transducing molecules (e.g., signaling by glucagon or β-adrenergic agonists), the full picture of metabolism-based signaling is not yet available. This is true in part because we are just beginning to realize that GSIS is likely to involve more than one, and perhaps several, signaling events that converge to activate exocytosis of insulin-containing secretory granules. The picture becomes even more complex when one considers the myriad of permissive, potentiating, and inhibitory agents that modulate the effects of glucose on insulin secretion.

In light of this growing appreciation of the complexity of the problem, we and others have realized that an interdisciplinary approach may be required to fully understand β-cell function. The result is that a field that began with the application of tools of metabolic biochemistry, enzymology, and physiology, now welcomes investigators applying molecular biology, gene discovery, cell and developmental biology, and biophysical chemistry strategies. In this study, we present our version of a multidisciplinary approach to the understanding of GSIS, and summarize the insights that we have gained to date and hope to gain in the future.

DEVELOPMENT OF TOOLS FOR STUDYING β-CELL FUNCTION

Recombinant adenovirus as a gene delivery tool. The use of a recombinant adenovirus for gene transfer into islet cells was first demonstrated in our laboratory in the early 1990s (1,2). Using this approach, genes can be delivered to isolated rat islets with a transfer efficiency of 70–80%, and to insulinoma cell line models with an efficiency approaching 100% (1–3). This level of efficiency for gene transfer is required for testing the impact of specific genes on candidate signaling pathways, and has led to an approach in which genes encoding various enzymes or other metabolic regulatory proteins are introduced into islets or insulinoma cell lines to specifically alter segments or branches of metabolic pathways. In some cases, this has resulted in potentially important insights into β-cell function (4,5).

Glucose-responsive and -unresponsive cell lines. A second important tool for our work is the development of a new set of cell lines for studying insulin secretion. The starting point for this work was the rat insulinoma cell line INS-1, which has been widely used in the field because it responds to glucose over a physiological range of concentrations (6). We isolated a large number of independent cell lines from the original INS-1 population, using a stable transfection strategy (7), and screened each clone for its ability to respond to a rise in media glucose from 3 to 15 mmol/l. As shown in Fig. 1, 70% of the clones isolated in this fashion were very poorly or not at all responsive to glucose, 20% of the clones were moderately responsive, and a few clones were robustly responsive, exhibiting 10-

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DMM, dimethylmalate; GSIS, glucose-stimulated insulin secretion; K_{ATP} channel, ATP-sensitive potassium channel; MCD, malonyl-CoA decarboxylase; NMR, nuclear magnetic resonance; PAA, phenylacetic acid; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase complex; TCA, tricarboxylic acid.

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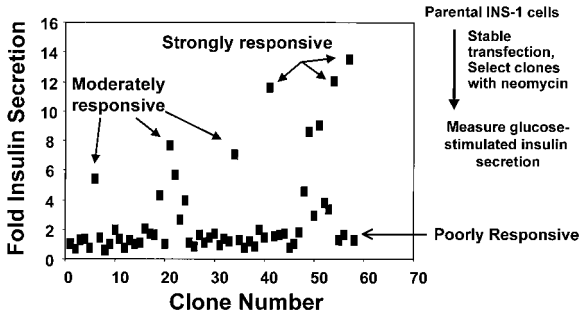

FIG. 1. Clonal heterogeneity in INS-1–derived cell lines. Parental INS-1 cells were stably transfected with a plasmid containing the human proinsulin gene, as previously described (14). After antibiotic selection, individual colonies were isolated and expanded and screened by measuring the fold increase in insulin secretion at 15 vs. 3 mmol/l glucose. Data for 58 individual clones are presented, and represent the mean of three independent measurements per clone.

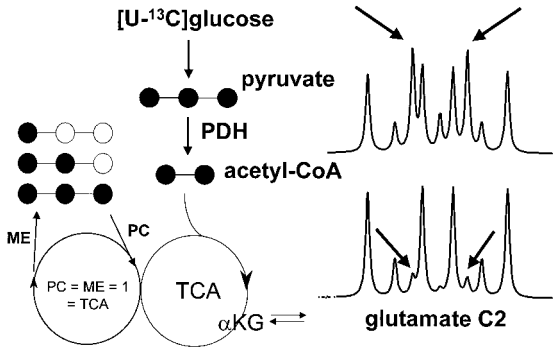

FIG. 2. Predicted NMR spectra in islet cells during stimulation with [U-^{13}C]glucose. The predicted NMR spectra of glutamate carbon 2 (C2) are shown for two metabolic extremes. The bottom spectrum is predicted if all [U-^{13}C]-labeled pyruvate derived from [U-^{13}C]glucose enters the TCA cycle via the PDH reaction. The top spectrum is predicted if pyruvate enters via both PDH and PC and there is active cycling between four carbon intermediate pools and pyruvate (the spectrum shown would appear if flux through the malic enzyme [ME] and PC equaled the TCA cycle flux). Note that in this second scenario, pyruvate can become labeled with ^{12}C (○) or ^{13}C (●). The two spectra contain the same peaks, but certain peaks are clearly much larger in the upper spectrum (designated by arrows). Integrated analysis of these kinds of spectral differences among all the carbons of glutamate allows the metabolic fate of pyruvate to be defined (18–22). αKG, α-ketoglutarate.

to 15-fold increases in GSIS. Further study of highly responsive cell lines such as 832/13 has revealed that these clones retain a long-term functional stability, extending for at least 7.5 months of continuous culture (7). Isolation of this new set of clones has allowed us to conduct biochemical studies in a phenotypically stable background. However, their greater value may ultimately be the opportunity for genetic and biochemical comparison of cell lines that differ in one important functional characteristic, GSIS, thereby allowing key mechanistic determinants to be identified.

Nuclear magnetic resonance–based analysis of glucose metabolism in insulin secreting cell lines. Traditional metabolic analysis of pancreatic islets has involved either the measurement of the concentration of intracellular or secreted intermediates via enzyme-based assays or the use of radioisotopically labeled metabolic fuels to measure the rate of a specific sequence of metabolic reactions. Although application of these techniques has been informative, a deeper understanding of GSIS mechanisms may require a more global or comprehensive "fingerprint" of metabolic changes that occur during glucose stimulation of β-cells.

One emergent technology that can be applied for this purpose is nuclear magnetic resonance (NMR)-based analysis of the metabolism of fuels that are labeled with heavy isotopes such as ^{2}H or ^{13}C. For example, we recently used $^{2}H_2O$ to trace the origin of glucose molecules contributing to glycogen synthesis in liver cells that contain overexpressed metabolic regulatory genes (8). With regard to β-cell metabolism, the question that we have addressed is the metabolic fate of pyruvate in mitochondria during GSIS. Pyruvate can enter the tricarboxylic acid (TCA) cycle via two prominent pathways. The pyruvate dehydrogenase complex (PDH) catalyzes the conversion of pyruvate to acetyl-CoA. An alternate entry point is the pyruvate carboxylase (PC)-catalyzed conversion of pyruvate to oxaloacetate. Several laboratories have reported on the surprisingly abundant expression of PC in β-cells, and it has been estimated that 40–50% of the pyruvate generated during glucose stimulation of β-cells enters mitochondrial metabolism via this route (9–17). In liver, PC pairs with PEPCK to catalyze early steps in gluconeogenesis. In islets, PEPCK is lacking, suggesting that PC-catalyzed conversion of pyruvate to oxaloacetate may have a specific signaling function in β-cells.

Anaplerotic influx of pyruvate into the TCA cycle may be linked to efflux of other intermediates from the mitochondria, including malate (16) or citrate (17), resulting in the synthesis of important coupling factors. Cytosolic malate can be reconverted to pyruvate via the malic enzyme, completing a pyruvate-malate cycle. An alternate cycle occurs when citrate leaves the mitochondria to be cleaved to acetyl-CoA and oxaloacetate by citrate lyase. The oxaloacetate formed via citrate cleavage can in turn be converted to malate via cytosolic malate dehydrogenase activity, and then back to pyruvate via malic enzyme to complete a pyruvate-citrate cycle. A cofactor common to the pyruvate-malate and pyruvate-citrate cycles is NADPH, which is produced as a by-product of the malic enzyme (12).

Over the course of the past several years, NMR-based techniques have been developed for determining the relative contribution of the PDH and PC reactions to pyruvate entry into the TCA cycle (18–22). These have involved the culture of cells in the presence of [U-^{13}C]glucose, and the subsequent extraction of cellular glutamate for NMR-based mass isotopomer analysis. Glutamate is the analyte chosen in these studies because it is present in high concentrations in β-cells, it is in equilibrium with the TCA cycle intermediate α-ketoglutarate, and its NMR signal is well isolated from those of other metabolic intermediates. Figure 2 illustrates the NMR spectra generated for carbon 2 of glutamate under vastly different metabolic circumstances. In the first scenario, 100% of the ^{13}C-labeled glucose is converted to pyruvate, and all of the pyruvate enters the TCA cycle via the PDH reaction. In an opposite scenario, the PDH and PC reactions each contribute equally to pyruvate entry into the TCA cycle and there is an active pyruvate-cycling pathway (as given by equal and high flux through the malic enzyme and PC). Certain peaks in the spectra are clearly different in the two circum-

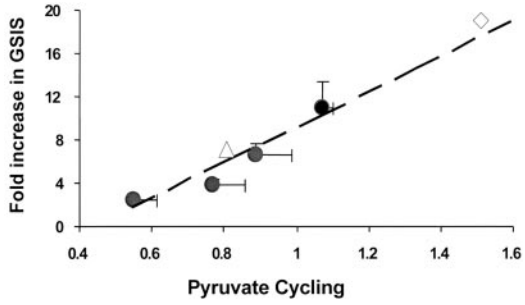

FIG. 3. Linear relation between GSIS and pyruvate cycling. ●, INS-1–derived cell lines 832/1, 832/2, 834/40, and 832/13 INS-1 cells in order of increasing capacity for GSIS (22); ◇, 832/13 cells incubated with 12 mmol/l glucose plus 10 mmol/l DMM, a stimulatory metabolite for pyruvate cycling; △, 832/13 cells incubated with 12 mmol/l glucose plus 5 mmol/l PAA, an inhibitor of PC and pyruvate cycling. Taken from Lu et al. (22), with permission.

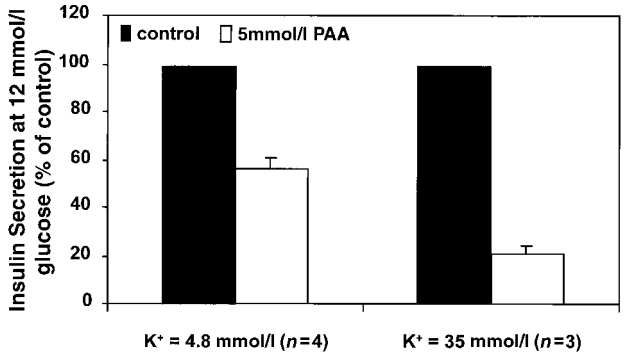

FIG. 4. Potent effect of PAA on the K_{ATP} channel–independent pathway of GSIS. Insulin secretion was measured during stimulation of INS-1–derived 832/13 cells (7) with 12 mmol/l glucose for 4 h. *Left panel*: secretion was measured in the presence of normal K^+ concentrations (4.8 mmol/l); *right panel*: experiments were conducted at 35 mmol/l K^+ to reveal the K_{ATP} channel–independent pathway. Note the much larger inhibitory effect of the PC inhibitor PAA in the latter group of cells.

stances, as is indicated by the arrows in Fig. 2. Similar diagnostic features are observed in the spectra for other glutamate carbons, and a comprehensive analysis of all of these differences leads to an estimate of the relative contributions of the different pathways (17–22). In what follows, we present discrete examples of what can be learned from the combined application of each of the foregoing tools.

APPLICATION OF NMR-BASED METABOLIC ANALYSIS TO GLUCOSE-RESPONSIVE AND -UNRESPONSIVE CELL LINES

The idea that mitochondrial metabolism of glucose generates important signals for insulin secretion has been supported by various kinds of experiments for many years (23–25). However, the specific metabolic pathways in mitochondria that participate in the generation of coupling factors for insulin secretion remains uncertain.

The approach that we have taken to this problem is apply the tool of NMR-based metabolic analysis to our robustly and poorly glucose-responsive INS-1–derived cell lines (22). Four independent cell lines with clearly distinguishable capacities for GSIS were incubated with varying concentrations of [U-^{13}C]glucose, followed by NMR-based mass isotopomer analysis of extracted glutamate. Measurements of GSIS were performed on the same set of cells used for NMR analysis. As shown in Fig. 3, we observed a tight correlation between PC-catalyzed pyruvate cycling activity and GSIS in the four cell lines. In contrast, entry of pyruvate into the TCA cycle via PDH did not correlate with glucose sensing (22).

To further investigate the relation between pyruvate cycling and GSIS, we measured the effects of stimulators and inhibitors of this pathway. First, we tested the cell permeant ester of malate, dimethylmalate (DMM), reasoning that this agent should increase pyruvate cycling by directly stimulating flux through the malic enzyme. The addition of DMM to our cell line most responsive to glucose, 832/13, nearly doubled insulin secretion at 12 mmol/l glucose and caused an increase in pyruvate cycling as measured by NMR (22). As a second test, phenylacetic acid (PAA), a well-known inhibitor of PC (17,26), was added to 832/13 cells in the presence of stimulatory glucose (12 mmol/l), resulting in a 50% inhibition of insulin secretion and a parallel decrease in pyruvate cycling (22). Remarkably, the data points created for 832/13 cells treated with DMM or PAA fell on the line relating pyruvate cycling and GSIS for the four independent INS-1–derived cell lines (Fig. 3). Thus, under both stimulatory (DMM) and inhibitory (PAA) conditions, there was a linear relation between GSIS and pyruvate cycling as measured by NMR.

The foregoing studies provide support for the idea that PC-catalyzed pyruvate cycling is an important pathway for the generation of coupling factors that mediate GSIS. Interestingly, neither the pyruvate-malate nor the pyruvate-citrate cycles produce ATP in a direct sense. Thus pyruvate cycling pathways may complement or augment signaling that occurs via the classic pathway of closure of ATP-sensitive K^+ (K_{ATP}) channels, secondary to a glucose metabolism–induced rise in the ATP:ADP ratio (24). One possibility is that pyruvate cycling is primarily responsible for insulin secretion via the K_{ATP} channel–independent pathway of insulin secretion. This pathway came to light in studies in which GSIS was shown to be clearly operative, even when regulation of K_{ATP} channels was prevented by exposure of cells to high concentrations of K^+ and the pharmacological agent diazoxide (27,28). That PC-catalyzed pyruvate cycling may be playing a role in the regulation of the K_{ATP}-dependent pathway is supported by the data in Fig. 4. As discussed earlier, incubation of robustly glucose-responsive 832/13 cells with the PC inhibitor PAA under normal ionic conditions (K^+ = 4.8 mmol/l) reduces insulin secretion by close to 50%. However, when the cells are studied at high K^+ (35 mmol/l), insulin secretion is inhibited by >80%. These results suggest that signals generated by PC-catalyzed pyruvate cycling may be particularly important in the regulation of the K_{ATP} channel–independent pathway of GSIS.

Although our studies cast new light on the potential importance of PC-catalyzed anaplerosis and pyruvate cycling in the generation of stimulus/secretion coupling factors, several important questions remain to be answered. First, the specific coupling factor(s) generated by anaplerotic pathways and the efflux of excess TCA cycle substrates remain to be identified. Possibilities that have

been proposed include NADPH formed as a by-product of the malic enzyme reaction or other NADPH-producing enzymes (12), malonyl-CoA formed as a by-product of citrate cleavage (29,30), or glutamate formed from α-ketoglutarate (31). Arguments for (31) and against (32) the idea that glutamate is a key coupling factor have been presented. Our own studies with NMR have failed to detect a rise in glutamate concentration during glucose stimulation of 832/13 cells, and therefore do not lend clear support to the glutamate model (22). Similarly, malonyl-CoA has been proposed as a coupling factor via its capacity to inhibit fatty acid oxidation and increase the levels of fatty acyl-CoAs (29,30). However, recent studies from our laboratory in which a recombinant adenovirus was used to express malonyl-CoA decarboxylase (MCD) resulted in a complete block of the normal glucose-induced rise in malonyl-CoA levels, but had no effect on GSIS (33). These studies were originally conducted in parental INS-1 cells using a goose MCD cDNA (34), but the results were subsequently confirmed in the robustly glucose-responsive 832/13 cell line with a human MCD clone engineered for targeting to the cytosolic compartment (33). However, it remains possible that glutamate or malonyl-CoA plays an important signaling role in the normal islet, but that such pathways are not essential in the INS-1–derived cell lines. Clearly more work will be required to resolve remaining disagreements and identify the relevant factor.

A second important area for further study is to determine if pyruvate cycling activity is altered in models of β-cell dysfunction, such as in islets subjected to chronic culture in high fat and/or glucose or in various animal models such as the ZDF rat. Studies of this nature are ongoing in our laboratory. Finally, we are currently applying the tools of gene discovery and recombinant adenovirus to identifying genes that may be used to enhance pyruvate-cycling activity, possibly leading to targeted strategies for improving glucose responsiveness in surrogate islet cells destined for transplant or in patients with type 2 diabetes.

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