
Perspectives in Diabetes

While Tinkering With the β -Cell. . . Metabolic Regulatory Mechanisms and New Therapeutic Strategies

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A common feature of the two major forms of human diabetes is the partial or complete loss of insulin secretion from β -cells in the pancreatic islets of Langerhans. In this article, we review the development of a set of tools for studying β -cell biology and their application to understanding of fuel-mediated insulin secretion and enhancement of β -cell survival. Insights into these basic issues are likely to be useful for the design of new drug and cell-based diabetes therapies. *Diabetes* 51: 3141–3150, 2002

The pancreatic islets of Langerhans play a critical role in regulation of fuel homeostasis by secreting insulin and glucagon in a regulated fashion. Secretion of these hormones becomes dysregulated in both major forms of diabetes, via destruction of insulin-producing β -cells in type 1 diabetes and loss of normal regulation of insulin secretion in type 2 diabetes. Development of the next generation of therapeutic strategies for diabetes, including cell-based insulin replacement in type 1 diabetes or drug therapies for enhancing insulin secretion in type 2 diabetes, will depend on a detailed understanding of the molecular and biochemical mechanisms involved in islet β -cell function and survival.

In light of the inadequacies of insulin injection therapy, great effort has been expended over the past 30 years in the development of cell-based insulin-replacement strategies. The fundamental concept is that transplantation of pancreatic islets might allow better regulation of insulin

delivery to diabetic patients (1). Very recently, a breakthrough occurred in this field in the form of a series of human islet transplants done by Shapiro et al. (2) at the University of Edmonton. In this study, patients received ~800,000 human islets, culled from two to three pancreases per recipient, via injection into the portal vein. Patients also received a cocktail of mild, nonsteroidal immunosuppressive agents. This resulted in the impressive finding of insulin independence in seven consecutive patients over an average time of 12 months. This compares to the prior 10 years of experience with human islet transplantation, in which success, defined as insulin independence 1 year after transplant, was reported in only 8% of patients (3).

While there is no question that the Edmonton Trial represents a major advance, several difficult issues must be overcome before cell-based insulin replacement can be broadly applied. First, the number of human pancreases that become available for islet harvesting in the U.S. is on the order of several thousand per year. When one considers that there are ~1 million patients with type 1 diabetes in the U.S., the disparity between supply and demand becomes clear. Another important issue is the difficulty inherent in controlling fundamental variables such as insulin content, insulin secretion, and cell viability in human islet preparations. Finally, immunoprotection of transplanted cells remains an issue, despite the dramatic success of the Edmonton trial. This is because the long-term effects of generalized, systemic immunosuppression, even with a mixture of relatively mild agents, won't be known for several years. It is also unclear whether such generalized immunosuppression will be an appropriate therapy for children with type 1 diabetes. Therefore, continued development of more specific and targeted methods for immunoprotection of transplanted cells is clearly needed.

The fundamental approach taken by our laboratory for solving these very difficult problems is to try to develop a replenishable source of cells that can deliver insulin in a regulated fashion and to find ways of protecting such cells in the transplant setting. Possible cell sources include stem cells or, alternatively, immortalized versions of the

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DMM, dimethylmalate; GSIS, glucose-stimulated insulin secretion; IFN- γ , interferon- γ ; IL-1 β , interleukin-1 β ; iNOS, inducible nitric oxide synthase; NF- κ B, nuclear factor κ B; NMR, nuclear magnetic resonance; PAA, phenylacetic acid; PBMC, peripheral blood mononuclear cell; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; RDA, representational difference analysis; TCA, tricarboxylic acid.

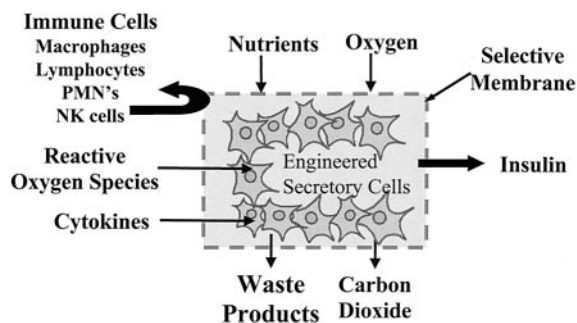


FIG. 1. Concept schematic: cell-based insulin replacement in diabetes using an encapsulated, expandable population of insulin secreting cells. The concept has the following three essential components: 1) an expandable population of cells engineered for appropriate regulation of insulin secretion by normal physiologic cues; 2) a retrievable macroencapsulation device that is rigorously cell impermeant but that allows entry and exit of nutrients, insulin, and other small molecules; 3) development of cells that are resistant to the cytotoxic effects of small, soluble mediators of the immune response, such as inflammatory cytokines and reactive oxygen species.

pancreatic islet β -cell. Over the course of the past 15 years, our group and its collaborators have developed a conceptual plan for cell-based insulin replacement. As summarized in Fig. 1, this plan has three fundamental elements. The first is to use the tools of genetic engineering to create an expandable population of secretory cells that will deliver large amounts of insulin in response to appropriate physiological cues. The second element is a macro-encapsulation membrane or device that allows safe transplantation of the engineered cells into diabetic subjects. This device should be selective, in that it should allow rapid diffusion of nutrients, oxygen, and waste products and, of course, the rapid exit of insulin. The device should also be rigorously cell exclusionary, such that it prevents contact of the transplanted cells with host tissues, while at the same time preventing contact of the cellular components of the host immune system, such as macrophages and lymphocytes, with the transplanted cells. However, a device of such design is unlikely to be sufficient to fully protect transplanted cells, because small soluble mediators of immune destruction such as inflammatory cytokines and reactive oxygen species will gain entry through the device membrane. Therefore, the last component of the concept is to develop methods for protecting the transplanted cells against the effects of these small soluble toxins.

Our work on cell-based insulin replacement has involved three groups: our academic laboratory at the University of Texas Southwestern Medical Center and its collaborators; a biotechnology company that we formed in Dallas, BetaGene, Inc.; and a materials engineering group, Gore Hybrid Technologies, founded by Dr. Mark Butler. With regard to the encapsulation device technology, Dr. Butler and his team have developed a retrievable and selective device fulfilling the criteria just summarized (4,5). However, the focus of this article will be on new advances in the understanding of islet biology.

In seeking to achieve the goal of cell-based insulin therapy, our group has worked with a variety of human and animal cell lines. We believe that an expandable source of human rather than animal cells will be required for success in cell-based therapy of human diabetes. One

approach to the development of human cells at BetaGene was to use human neuroendocrine cell lines for insulin delivery. In these studies, expression of the human proinsulin gene in a human lung neuroendocrine cell line resulted in secretion of correctly processed insulin in response to different agents like carbachol or the phorbol ester, PMA, but not in response to the key physiologic regulator, glucose (H. Hohmeier, T. Becker, A. Thigpen, et al., unpublished observations). In short, while it has been possible to create rodent cell lines that exhibit robust glucose-stimulated insulin secretion (see below), development of human cells that stably maintain this property has been much more elusive.

The difficulty associated with procurement of stable human cell lines for insulin replacement has prompted us in recent years to refocus our efforts toward gaining a deeper understanding of the genetic and biochemical mechanisms of β -cell function. Our work on β -cell biology has evolved such that we now utilize several technologies in an integrated fashion to gain insights into mechanisms of fuel-regulated insulin secretion and methods for enhancing β -cell survival. This includes novel cell models, techniques of gene discovery and genetic engineering, and, very recently, the application of nuclear magnetic resonance (NMR) for metabolic analysis of insulin-producing cells. Note that insights gained in these areas are also likely to be relevant to the understanding of β -cell dysfunction in type 2 diabetes, possibly leading to development of new therapeutic strategies for enhancing β -cell mass or performance in this disease.

DEVELOPMENT OF TOOLS FOR STUDYING β -CELL FUNCTION

A. Recombinant adenovirus as a gene delivery tool.

One very important need in our research is to be able to test the functional impact of genes in a rapid and efficient fashion. The use of recombinant adenovirus for gene transfer into islet cells was first demonstrated in our laboratory in the early 1990s (6,7). Figure 2 shows that adenovirus-mediated transfer of the β -galactosidase gene into isolated rat islets results in expression of the gene in 70–80% of the islet cells (6). This compares very favorably to traditional techniques such as electroporation, which only allow gene transfer efficiencies on the order of 10–20% in isolated islets, which is inadequate for testing hypotheses about stimulus/secretion coupling pathways. Our success in obtaining rapid and efficient transfer of genes into islets and β -cell lines has allowed us to prepare and test a number of viruses containing genes relevant to β -cell signaling (rev. in 8–10), some of which will be discussed in more detail below.

B. Glucose responsive and unresponsive cell lines.

A second important tool for our work is a new cellular model for studying insulin secretion. The start-point for this work was a pre-existing rat β -cell line, INS-1, created by Claes Wollheim and associates in Geneva (11). This cell line has been widely used in the field of β -cell biology because it secretes insulin in response to physiological glucose concentrations. However, two problems with these particular cells have emerged. One is that insulin secretion is stimulated by only three- to fourfold as the glucose concentration is raised from 3 to 15 mmol/l (11–13). This is far less

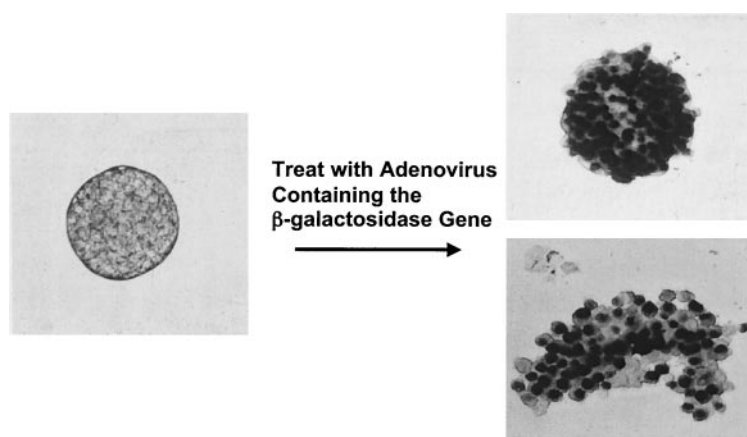


FIG. 2. Use of recombinant adenovirus for gene transfer in isolated pancreatic islets. The panel at left shows a typical single rat islet, consisting of cluster or several hundred cells. Following treatment with a recombinant adenovirus containing the β -galactosidase gene (AdCMV- β GAL) and staining of the treated islets with a chromogenic substrate, a large number of the constituent cells of the islet are stained blue, indicating successful gene transfer. See refs. 6–8 for details.

than that seen for freshly isolated pancreatic islets, which exhibit a 15-fold response to a similar increment in glucose concentrations. A second problem is that with continued growth of the cells, even the three- to fourfold response is lost as a function of time in culture.

In an attempt to address these issues, we isolated a large number of independent clonal cell lines from the parental INS-1 stock, using a stable transfection strategy (14). Once the clones were available, they were expanded and screened for their capacity for glucose-stimulated insulin secretion (GSIS). Figure 3 summarizes this screen of 58 independent INS-1-derived clones, expressed as fold-stimulation of insulin secretion as glucose is raised from 3 to 15 mmol/l. Fully 70% of the clones isolated in this fashion are very poorly glucose responsive or not responsive to glucose at all. Another subgroup of \sim 20% of the clones are moderately responsive and a few clones are robustly responsive with 10- to 15-fold increases in insulin secretion in response to high glucose. Further study of clones of the highly responsive class, such as line 832/13, reveals that these clones retain a long-term functional stability, such that robust glucose responsiveness can be demonstrated after 9 months or more of continuous culture (14). This observation is important for three reasons. First, the development of cell lines such as 832/13 provides us with a stable model for probing the biochemical mechanisms involved in control of insulin secretion. Second, procurement of cell lines that are truly clonal allows maintenance of reliable and predictable functional characteristics. Our findings in this regard are consistent with the work of others in the field, notably Knaack et al. (15), who have developed stable mouse cell lines by dilution cloning strategies. This type of functional consistency will be a critical component of any cell-based insulin-replacement therapy and will be required for acceptance of these strategies by regulatory agencies and patients. Finally, the isolation of stably glucose responsive and unresponsive cell lines from a single starting source allows us to apply genetic and biochemical approaches for comparing these two discrete populations, thereby allowing key mechanistic determinants of robust GSIS to be identified, as detailed below.

C. Development of a selection strategy for isolation of cytokine-resistant insulinoma cell lines. Immunological attack on islet β -cells in type 1 diabetes involves direct interaction of islet cells and immune cells, as well release of a complex mixture of toxic soluble factors such

as inflammatory cytokines and reactive oxygen species (16–20). A key obstacle that must be overcome for development of cell-based insulin-replacement strategies is the development of cells that are resistant to cell killing via the latter mechanisms. To this end, we recently developed a selection strategy for isolating cell lines that are resistant to the cytotoxic effects of multiple cytokines (21). This involved incubation of INS-1 cells in the combined presence of gradually increasing doses of interleukin-1 β (IL-1 β) and interferon- γ (IFN- γ), culminating with the isolation of a population of cells able to survive in the presence of very high concentrations of these cytokines. As shown in Fig. 4, unselected INS-1 cells exhibited \leq 20% viability after 48 h of treatment with 10 ng/ml IL-1 β + 100 units/ml IFN- γ or with conditioned media from activated rat peripheral blood mononuclear cells (PBMCs). Following the selection procedure, cells were completely resistant to the cytotoxic effects of the cytokine mixture or media from conditioned PBMCs. Note that the PBMC media contains cytokines and toxic factors in addition to IL-1 β and IFN- γ , but the cells still remain fully protected. The protective effect is only partially dependent on the continual presence of cytokines in the culture medium. Thus, cells taken through the 8-week selection protocol and then cultured for an additional 2 months in the absence of cytokines remain 70% viable following 48 h of exposure to IL-1 β + IFN- γ , as compared with only 20% in the unselected controls (Fig. 4). Studies on the molecular

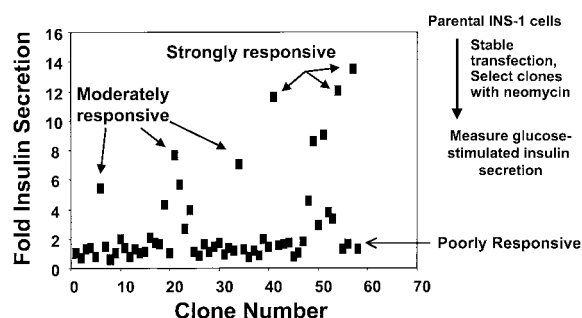


FIG. 3. Clonal heterogeneity in INS-1-derived cell lines. Parental INS-1 cells were stably transfected with a plasmid containing the human proinsulin gene as described in ref. 14. Following antibiotic selection, individual colonies were isolated, expanded, and screened by measuring the fold increase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

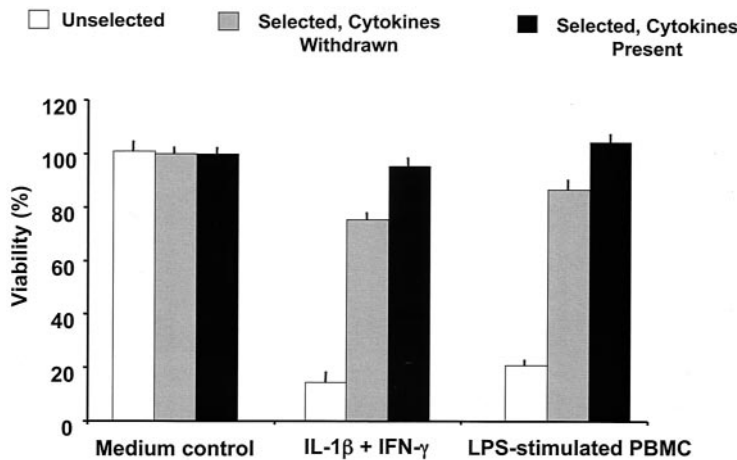


FIG. 4. Selection of INS-1-derived cells resistant to the cytotoxic effects of inflammatory cytokines and other agents. Three groups of cells were studied: 1) INS-1 cells subjected to a selection procedure involving continual growth in IL-1 β + IFN- γ for 8 weeks (Selected, cytokines present); 2) INS-1 cells subjected to the 8-week selection procedure followed by culture in the absence of cytokines for a period of 2 months (Selected, cytokines withdrawn); and 3) INS-1 cells grown in normal medium with no selection (Unselected). Details of these procedures are provided in ref. 21. The three cell groups were then exposed to 10 ng/ml IL-1 β + 100 units/ml IFN- γ (IL-1 β + IFN- γ) or to conditioned media from LPS-treated peripheral blood mononuclear cells (LPS-stimulated PBMC) for 48 h. Cell viability was then measured using the MTT assay.

basis for cytokine resistance in the selected cell lines are summarized later in this article. In summary, this new model allows us to gain insight into the genes and biochemical pathways that are responsible for cytokine-mediated β -cell killing, possibly leading to development of methods for protecting transplanted insulin-secreting cells.

RECOMBINANT ADENOVIRUSES APPLIED TO GAINING INSIGHT INTO STIMULUS/SECRETION COUPLING MECHANISMS IN β -CELLS: AN EXAMPLE

Glucose causes insulin to be released from β -cells via the generation of signals induced through metabolism of the sugar (22,23; Fig. 5). Glucose enters the pancreatic islet β -cell via facilitated glucose transporters, primarily GLUT-2 in rat islets. Once inside the cell, glucose is converted to glucose-6-phosphate in the glucokinase reaction, and the glucose-6-phosphate then enters glycolysis to be converted to pyruvate, with some ATP produced as a byproduct. Once pyruvate is generated it can enter the mitochondria, where it flows into the tricarboxylic acid (TCA) cycle at several points. Oxidation of pyruvate in the TCA cycle results in further production of ATP. A third way by which ATP can be produced during glucose metabolism is via various reducing equivalent shuttles, where the reducing power of NADH in the cytosol is converted to reducing power in the mitochondria (23–27).

Metabolic pathways that generate ATP have received major attention from islet biologists because a rise in the ATP-to-ADP ratio has been shown to inhibit ATP-sensitive K⁺ (K_{ATP}) channels in β -cells, resulting in activation of voltage-gated calcium channels (28). The resultant influx of extracellular calcium causes the activation of certain protein kinases and other enzymes, leading to exocytosis of insulin from preformed secretory granules. While regulation of K_{ATP} channels is undoubtedly an important signal for regulation of insulin secretion, it is clearly not the only mechanism. Various laboratories have shown that GSIS still occurs when the ATP-sensitive K⁺ channel is taken out of play by pharmacologic agents or the application of very high concentrations of potassium to the cell (29,30). However, the biochemical mechanism of this so-called “K_{ATP} channel-independent” pathway of glucose sensing remains to be defined.

The general approach that our group has taken for

unraveling this complex network of metabolic signaling in the β -cell is to use the recombinant adenovirus tool described earlier to introduce genes that perturb metabolism in defined ways. An example of this approach will be highlighted (12). The carbohydrate fuel, glycerol, is not metabolized in β -cells and is therefore unable to stimulate insulin secretion. β -Cells are unable to metabolize glycerol because they lack the first enzyme of glycerol metabolism, glycerol kinase (12). These observations raised the following set of questions: 1) Does adenovirus-mediated expression of glycerol kinase allow glycerol to be metabolized, and does it lead to glycerol-stimulated insulin secretion? and 2) if the answer to the first question is yes, can we gain insight into the mechanisms by which insulin secretion is regulated by comparing the metabolic fates of glycerol and glucose?

The answer to the first question is clear from the data in Fig. 6. In these experiments, rat pancreatic islets were treated with a control adenovirus (AdCMV- β GAL) or a virus containing a bacterial gene encoding glycerol kinase (AdCMV-GlpK). Increasing the glycerol concentration from 0 to 5 mmol/l caused a sharp rise in insulin secretion in glycerol kinase-expressing cells, but not in control cells. However, upon exposure of cells to 20 mmol/l

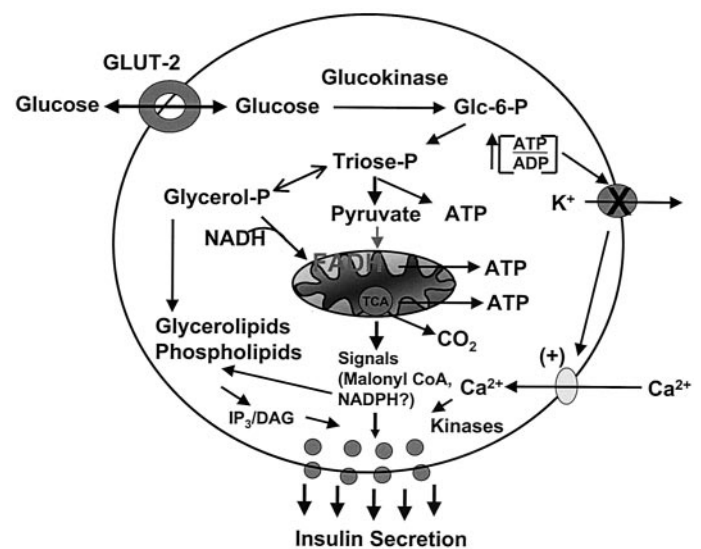
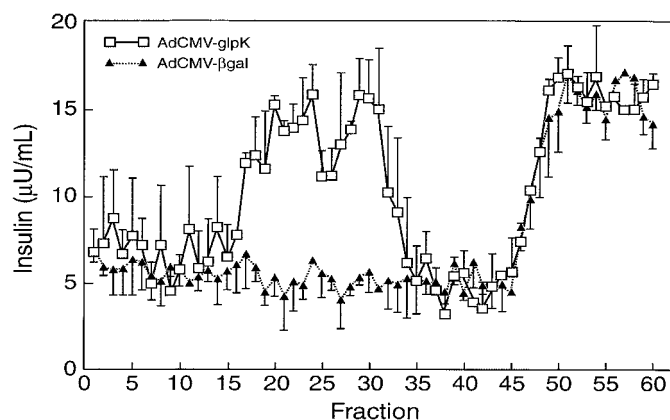


FIG. 5. Schematic summary of biochemical events implicated in GSIS.



Glycerol (mM)	0	5	0	5
Glucose (mM)	1		20	

FIG. 6. Adenovirus-mediated expression of glycerol kinase in rat islets confers glycerol-stimulated insulin secretion. Rat islets were treated with a virus containing the glycerol kinase gene (AdCMV-glpK) or, as a control, the β -galactosidase gene (AdCMV- β gal). Perfusion of such islet preparations revealed that only islets treated with AdCMV-glpK were able to increase insulin secretion in response to glycerol (9). Data reproduced from ref. 12 with permission.

glucose, potent stimulation of insulin secretion occurred in both groups. This experiment shows that it is possible to make glycerol equal in potency to glucose as a stimulator of insulin secretion simply by expressing glycerol kinase.

We next investigated the metabolic fate of glycerol in these cells. We first addressed whether the capacity of glycerol to serve as the backbone for esterification of fatty acids to make triglycerides is important for regulation of insulin secretion. This was done by monitoring the incorporation of radioactive glycerol into cellular lipids in the presence or absence of triacsin C, an inhibitor of long-chain acyl CoA synthetase. We observed that INS-1 cells engineered for glycerol kinase expression and incubated in the absence of triacsin C increased their incorporation of radiolabeled glycerol into lipids as a function of glycerol concentration. In contrast, triacsin C-treated cells exhibited a complete block in the incorporation of glycerol into the lipid pool. Despite the potent effects of the drug on lipid esterification, glycerol-stimulated insulin secretion remained intact, suggesting that the esterification of fatty acids into glycerol lipids is not an important metabolic pathway for controlling insulin release (12).

We continued the studies by comparing the metabolic fates of glycerol and glucose in glycerol kinase-expressing β -cells. We found that glycerol is more effectively converted to lactate than is glucose. Conversely, when we compared the oxidation of glucose and glycerol, glucose appeared to be the more efficiently oxidized fuel (12).

The following conclusions arise from these experiments. First, expression of glycerol kinase is clearly sufficient to confer glycerol-stimulated insulin secretion. This serves as added proof that regulation of insulin secretion by simple carbohydrate fuels is a metabolism-driven process, since without the expression of glycerol kinase there is no glycerol metabolism or regulation of insulin secretion by glycerol. The experiments just cited plus others that will not be summarized due to space limitations (13,31) also suggest that a fully active link between glucose and lipid metabolism is not required for glucose-stimulated insulin secretion. Last, glycerol stimulates insulin secretion despite its preferential conversion to lactate. This suggests that oxidation of these simple fuels may not be the critical metabolic pathway that regulates insulin release.

NMR-BASED ANALYSIS OF GLUCOSE METABOLISM IN INSULIN-SECRETING CELL LINES

The results of the glycerol kinase studies are somewhat surprising in light of good evidence from several laboratories that mitochondrial metabolism of glucose generates important signals for insulin secretion (32,33). This raises the issue of what actually happens to pyruvate as it enters the mitochondria and whether pathways other than the classical pathway of glucose oxidation may be important.

To answer this question, we have recently been comparing the glucose-responsive and -unresponsive INS-1-derived clones described earlier at two levels. The first is to analyze the metabolic differences between strongly responsive and poorly responsive lines, using the powerful technique of ^{13}C NMR. The second has been the identification of genes that are differentially expressed in the strongly responsive versus poorly responsive lines.

The application of NMR-based analysis of metabolic pathways has been made possible via a collaboration with Professor Dean Sherry of the Rogers NMR Center at UT Southwestern Medical Center, Dallas. Dr. Sherry and his colleague, Dr. Craig Malloy, have developed NMR-based methods for analyzing metabolism of pyruvate in mitochondria (34–37). As shown in Fig. 7, there are two sights of entry for pyruvate into the TCA cycle. The more familiar one is through the pyruvate dehydrogenase complex, which converts pyruvate to acetyl CoA. In β -cells, another important entry point for pyruvate is through the enzyme pyruvate carboxylase (PC), which converts pyruvate to oxaloacetate. The work of the groups of Michael MacDonald, Marc Prentki, and others has highlighted the abundant expression of PC in β -cells and has shown that this mode of entry into the cycle, known as anaplerosis, is an active metabolic event (38–46). In liver, PC pairs with phosphoenolpyruvate carboxykinase (PEPCK) to catalyze early steps in gluconeogenesis. In islets, PEPCK is lacking, suggesting that PC-catalyzed conversion of pyruvate to oxaloacetate has a specific function in β -cells distinct from that in liver. Radioisotopic methods have been used to estimate that 40% of the pyruvate generated during glucose stimulation of β -cells enters mitochondrial metabolism via PC-catalyzed conversion to oxaloacetate (42–45). It has

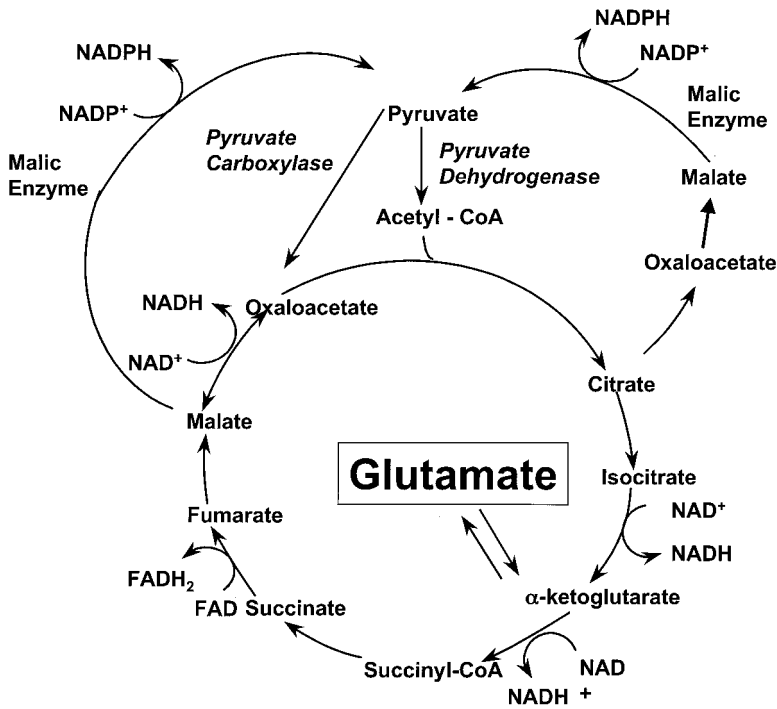


FIG. 7. Possible pathways of mitochondrial pyruvate metabolism in pancreatic islets. Pyruvate can enter the TCA cycle via the classical pathway of PDH-catalyzed conversion to acetyl CoA, but can also enter the cycle through pyruvate carboxylase. Because islets lack PEPCK activity, the pyruvate/malate or pyruvate/citrate cycling pathways shown could represent important pathways for generation of coupling factors for GSIS. NMR-based mass isotomer analysis of glutamate is used to distinguish the various metabolic fates of pyruvate following incubation of β -cells with $[U-^{13}C]$ glucose (49).

been further proposed that PC-catalyzed anaplerotic influx of pyruvate into the TCA cycle is linked to efflux of other intermediates from the mitochondria, including malate (45) or citrate (46), resulting in 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. Acetyl-CoA so formed can be converted to malonyl-CoA, which has been proposed as a coupling factor (17,48), although evidence against this idea has also been presented (13,31). 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 (Fig. 7). A cofactor common to both the pyruvate-malate and pyruvate-citrate cycles is NADPH produced as a byproduct of the malic enzyme (41).

The question that arises is whether one can discriminate between pyruvate dehydrogenase catalyzed entry of pyruvate into the TCA cycle versus PC-catalyzed entry and, if so, whether either one of these pathways has special significance for regulation of insulin secretion? The approach that we have taken to this problem is to incubate our robustly and poorly glucose responsive INS-1-derived cell lines with ^{13}C -labeled glucose, followed by extraction of glutamate from the cells and NMR analysis of its labeling pattern (49). Glutamate is the intermediate chosen for analysis because it is present at high concentrations in cells and it is in equilibrium with an intermediate of the TCA cycle, α -ketoglutarate.

While the NMR spectra generated can be quite complex, certain signature features allow us to track the mode of entry of pyruvate into the TCA cycle, as is illustrated by examination of the spectra predicted in two vastly different metabolic circumstances (Fig. 8). In the first scenario, we assume that 100% of the ^{13}C -labeled glucose is con-

verted to pyruvate and that all of the pyruvate enters the TCA cycle via the pyruvate dehydrogenase (PDH) reaction. In an opposite scenario, we assume that the PDH and PC reactions each contribute 50% of the pyruvate entry into the TCA cycle. Certain peaks in the NMR spectra are vastly different in the two metabolic circumstances, as indicated by the arrows in Fig. 8. Computer-based deconvolution of the NMR spectra for all carbons of glutamate allows one to gain an estimate of the relative contributions of the two pathways, using the methods developed by Malloy, Sherry, and colleagues (34–37).

As shown in Fig. 9, there is a remarkable correlation ($r = 0.92$) between PC-catalyzed pyruvate cycling activity and GSIS in four independent INS-1-derived cell lines with

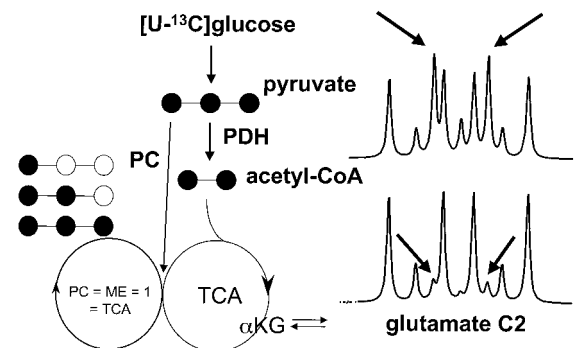


FIG. 8. Predicted NMR spectra in islet cells during stimulation with $[U-^{13}C]$ glucose. The predicted NMR spectra of glutamate carbon 2 (C2) are shown for two metabolic extremes. The bottom spectrum is predicted if all $[U-^{13}C]$ -labeled pyruvate derived from $[U-^{13}C]$ glucose enters the TCA cycle via the PDH reaction. The top spectrum is predicted if 50% of the pyruvate enters via PDH, and the other 50% enters via PC. Note that in this second scenario, pyruvate can become labeled with ^{12}C (○) or ^{13}C (●). Note that the two spectra contain the same peaks, but that certain peaks are clearly much larger in the upper spectrum (designated by arrows). Integrated analysis of these kinds of spectral differences among all of the carbons of glutamate allows the metabolic fate of pyruvate to be defined (34–37,49).

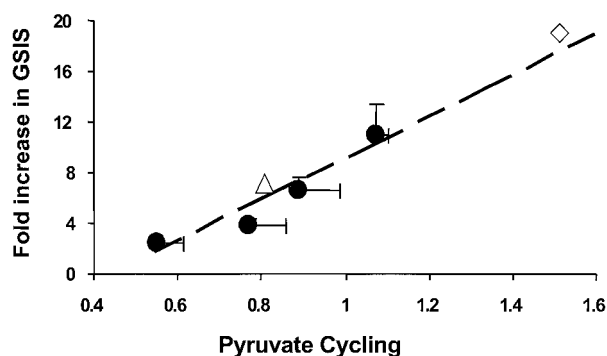


FIG. 9. Linear relationship between glucose-stimulated insulin secretion GSIS and pyruvate cycling. \circ , data from INS-1-derived cell lines 832/1, 832/2, 834/40, and 832/13 INS-1 cells in order of increasing capacity for GSIS (14,48). \diamond , 832/13 cells incubated with 12 mmol/l glucose plus 10 mmol/l dimethylmalate, a stimulatory metabolite for pyruvate cycling. \triangle , 832/13 cells incubated with 12 mmol/l glucose plus 5 mmol/l PAA, an inhibitor of pyruvate carboxylase and pyruvate cycling. Taken from ref. 49, with permission.

different degrees of glucose responsiveness. In contrast, entry of pyruvate into the TCA cycle via PDH does not correlate with glucose sensing (49).

To further investigate the relationship 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 anaplerosis and hence stimulate pyruvate cycling. Addition of DMM to our most glucose responsive cell line, 832/13, nearly doubled insulin secretion at 12 mmol/l glucose (49). Remarkably, this increase in insulin secretion was correlated with an increase in pyruvate cycling, such that the new data point fits precisely on the line relating pyruvate cycling and insulin secretion for the four variously responsive INS-1-derived cell lines (Fig. 9). As a second test, phenylacetic acid (PAA), a well-known inhibitor of PC (16,50), was added to 832/13 cells in the presence of stimulatory glucose (12 mmol/l), resulting in 50% inhibition of insulin secretion and a parallel decrease in pyruvate cycling of 25% (49). Again, the data point created for 832/13 cells treated with PAA falls on the line relating pyruvate cycling and GSIS for the four independent INS-1-derived cell lines (Fig. 9). Thus, under both stimulatory (DMM) and inhibitory (PAA) conditions, there is a linear relationship between GSIS and pyruvate cycling as measured by NMR.

One potential concern with regard to our conclusions about the link between pyruvate cycling and insulin secretion is that pyruvate cycling flux, as estimated by the NMR isotopomer method, is expressed as a ratio relative to TCA cycle flux. If absolute flux of pyruvate through PDH was different in glucose-responsive versus -unresponsive cells, this could mean that no real change in PC-catalyzed pyruvate cycling flux had actually occurred. If PDH-catalyzed glucose oxidation differed in the two cell lines, this should be reflected by a change in O_2 consumption. However, direct O_2 consumption measurements on actively respiring cells showed no difference between robustly or poorly glucose-responsive cells (49).

The following key summary points emerge from the NMR experiments. First, PC-catalyzed pyruvate cycling activity correlates with GSIS in INS-1-derived cell lines, while no correlation is observed between glucose respon-

siveness and PDH-catalyzed entry of pyruvate into the TCA cycle. Second, the PC inhibitor PAA impairs GSIS while preferentially inhibiting pyruvate cycling relative to TCA cycle influx. Third, a cell-permeant cycling intermediate, DMM, is a potent potentiator of GSIS in a glucose-responsive INS-1-derived cell line. These findings provide strong support for the idea that PC-catalyzed pyruvate cycling is a critical pathway for generation of coupling factors that mediate GSIS. Current work is focused on further exploration of this concept, with the following immediate goals: 1) to identify the coupling factor that links pyruvate cycling to exocytosis of insulin containing secretory granules; 2) to determine if pyruvate cycling activity is altered in models of β -cell dysfunction; and 3) to identify genes that may be used to enhance pyruvate cycling activity, possibly serving to improve glucose responsiveness of candidate human cell lines derived from stem cells or other sources.

EXPLOITATION OF INS-1-DERIVED CELL LINES FOR GENE DISCOVERY.

While biochemical comparison of glucose-responsive and -unresponsive INS-1-derived cell lines has been quite informative, a genetic comparison of these same lines could yield additional information about genes that participate in determining the fully differentiated functions of the mature β -cell. We have taken a three-step approach to achieving this goal. The first step has been to compare the expression levels of a set of candidate genes that includes key metabolic regulatory proteins such as GLUT2 and glucokinase, as well as various transcription factors implicated in β -cell development such as PDX-1, Nkx 2.2 and 6.1, and neuro D. The second approach has been to perform a subtractive hybridization procedure known as representational difference analysis (RDA) (51). This method has identified approximately a dozen genes that are preferentially expressed in glucose-responsive cell lines relative to poorly responsive lines and another dozen that are preferentially expressed in the poorly responsive lines relative to the strong responders (P. Jensen, C.B.N., unpublished observations). Finally, we have entered into a collaboration with a biotechnology company, CuraGen, Inc. (New Haven, CT), in which their GeneCalling technology (52) has been applied to our INS-1-derived cell lines. This analysis has identified a set of \sim 130 genes that discriminate our glucose-responsive and poorly responsive cell lines. We are now entering a phase of the analysis in which candidate genes identified by each of the three approaches are being prepared as recombinant adenoviruses to allow the effect of altered expression of individual genes to be assessed in the various INS-1-derived cell lines. This analysis is also likely to involve suppression of expression of certain candidate genes via antisense oligonucleotides or RNAi technologies.

IDENTIFICATION OF GENES THAT CONFER PROTECTION AGAINST CYTOKINE-INDUCED CYTOTOXICITY.

The final section of this article focuses on our efforts to identify genes involved in protection of β -cells against damage induced by inflammatory cytokines and other soluble toxins. The development of a selection method for

isolation of cytokine-resistant cell lines was summarized earlier. Cells with complete resistance to the cytotoxic effects of IL-1 β and IFN- γ were selected via an 8-week regimen of exposure to gradually increasing concentrations of these inflammatory cytokines (21).

Recent efforts have been focused on understanding the mechanisms by which the selection strategy has caused stable resistance to IL-1 β and transient but reinducible resistance to IFN- γ . Analysis of components of the known IL-1 β signaling pathway reveals that selected cell lines have a defect in activation of the transcription factor nuclear factor- κ B (NF- κ B) (21). Consistent with this finding, selected lines also fail to increase expression of inducible nitric oxide synthase (iNOS), the NF- κ B target gene. They also fail to increase nitric oxide production in response to acute exposure to IL-1 β , while unselected cell lines have large increases in both variables. More recently, in collaboration with the laboratory of Zhijian (James) Chen at UT Southwestern Medical Center, Dallas, we have determined that the defect in NF- κ B activation in selected lines occurs downstream of degradation of the NF- κ B regulatory protein, I κ B, since the rate of degradation of I κ B is identical in selected and unselected cell lines acutely exposed to IL-1 β . Although this narrows the search for the factor(s) involved in IL-1 β resistance in selected lines, the precise mechanism remains unknown and is the subject of ongoing investigation in our laboratory.

We have also investigated the mechanism by which IFN- γ -mediated cytotoxicity is impaired in the selected cell lines (21,53). The IFN- γ receptor is a member of the JAK/STAT receptor family. The JAK kinase activity associated with the IFN- γ receptor phosphorylates and activates the transcription factor STAT-1 α . When the STAT-1 α transcription factor is phosphorylated and dimerizes, it translocates to the nucleus where it acts upon several genes. As shown in Fig. 10, phosphorylation of STAT-1 α in response to acute cytokine exposure is similar in selected and unselected cell lines, but the total amount of STAT-1 α protein is dramatically increased in the selected cells (53). Furthermore, cytokine withdrawal, which results in loss of IFN- γ resistance in selected lines, also results in a fall in STAT-1 α expression to the same levels as those found in unselected cells. These data suggest that an increase in STAT-1 α expression may be a mechanism by which the cytokine selection procedure results in IFN- γ resistance.

To test this hypothesis, we prepared a recombinant adenovirus containing the STAT-1 α gene and demonstrated that treatment of unselected INS-1 cells with this virus caused a large increase in STAT-1 α protein expression (Fig. 11). The effect of this maneuver was as hypothesized—cells with overexpressed STAT-1 α gained resistance to IFN- γ -mediated cytotoxicity and, more importantly, to the combined effects of IL-1 β and IFN- γ (53). While this resistance was not complete, it should be noted that STAT-1 α was expressed in the background of unselected cell lines with no resistance to IL-1 β . Studies to investigate the effect of STAT-1 α expression in cell lines with stable resistance to IL-1 β are ongoing, as are experiments attempting to define the mechanisms by which high levels of STAT-1 α expression interfere with cytokine-

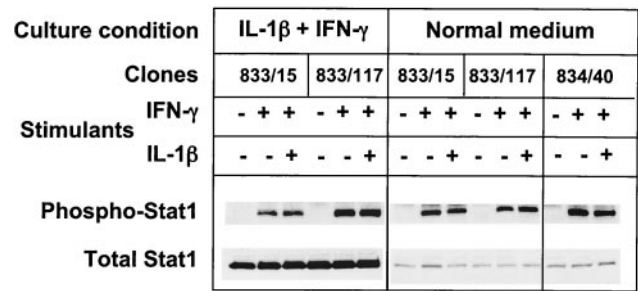


FIG. 10. STAT-1 α expression and phosphorylation in INS-1-derived cell lines. Unselected (833/40) and selected (833/15, 833/117) INS-1-derived clonal cell lines were grown in the presence or absence of IL-1 β + IFN- γ (100 units/ml and 10 ng/ml, respectively) for a period of 48 h (culture condition) in 12-well plates. Cells were then shifted to serum free medium without cytokines overnight, treated with or without the indicated concentrations of cytokines (stimulants) for 20 min, washed with PBS, and lysed. Fifty micrograms of total protein was loaded in each lane. Activated STAT-1 was detected by using anti-phospho-STAT-1 (Tyr701) antibody. Total STAT1 α was measured with an anti-STAT-1 antibody. The results show that resistance to IFN- γ and IFN- γ + IL-1 β -mediated cytotoxicity (achieved in the INS_{res}-derived lines only when cultured in cytokines) is correlated with a large increase in expression of STAT-1 α . Data taken from ref. 53, with permission.

induced cytotoxicity. Importantly, overexpression of STAT-1 α did not impair GSIS in robustly responsive INS-1-derived cell lines such as 832/13 (53). In conclusion, the resistance to IL-1 β in selected cell lines appears to involve impairment of NF- κ B signaling, while resistance to IFN- γ is due at least in part to upregulation of STAT-1 α . Further, expression of STAT-1 α in INS-1 cells confers cytokine resistance without causing impairment of GSIS. Thus, STAT-1 α emerges as a gene product that may be important in conferring protection against cytokine-induced damage of transplanted islet cells or that may be exploited in preserving β -cell mass in diabetes. However, much more remains to be learned about the mechanism of action of overexpressed STAT-1 α in prevention of cytotoxicity. We will also seek to learn more about other genes whose expression may be affected in our new model of cytokine resistance and β -cell survival.

SUMMARY AND FUTURE DIRECTIONS

A great deal has been learned about the islet β -cell in recent years via the application of the tools of molecular biology. Many laboratories, including our own, are engaged in gene discovery experiments using various models of β -cell differentiation, expansion, survival, and dysfunction, and the field will soon be confronted with lists of hundreds, even thousands, of genes that are differentially expressed in islet β -cells under different conditions. Identification of the small subset of these genes that constitute therapeutic targets in type 2 diabetes, or that can be exploited to encourage β -cell growth, differentiation, and survival in development of surrogate β -cells for therapy of type 1 diabetes are perhaps the next great challenges of this area of research. Ultimate success will require the very careful selection of appropriate cellular models, the use of interdisciplinary approaches for phenotypic analysis, and the ability to screen the functional impact of candidate genes in a rapid and reliable fashion.

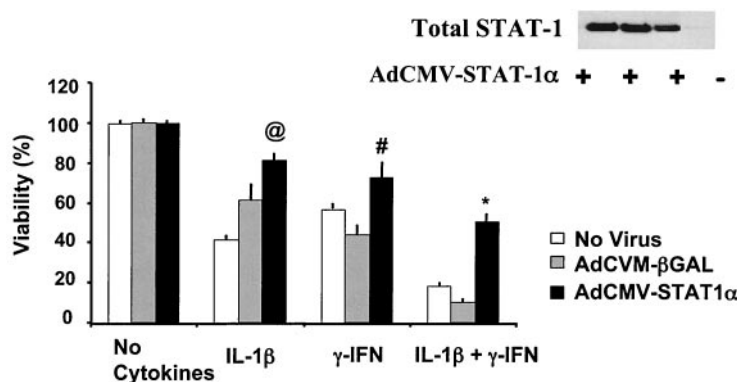


FIG. 11. Adenovirus-mediated expression of STAT-1 α in a cytokine-sensitive INS-1-derived cell line confers broad resistance to cytokine-induced cytotoxicity. The cytokine-sensitive cell line 834/40 was treated with AdCMV-STAT1 α , the same amount of AdCMV- β GAL virus, or no virus. These cell groups were cultured in normal medium for 24 h and were then treated with 100 ng/ml IL-1 β , 100 units/ml IFN- γ , or both cytokines for 48 h. Cell viability was then determined with the MTT assay, as described (53). The immunoblot shown in the upper right portion of the figure demonstrates overexpression of STAT-1 α in cells treated with the AdCMV-STAT1 α virus. Symbols refer to comparisons of viability of STAT-1 α overexpressing cells to other groups, as follows: [@] $P < 0.001$ versus untreated control, $P < 0.03$ vs. AdCMV- β GAL-treated control; [#] $P = 0.05$ vs. untreated control, $P < 0.007$ vs. AdCMV- β GAL-treated control; ^{*} $P < 0.002$ vs. either control group.

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