Glycerol-Stimulated Proinsulin Biosynthesis in Isolated Pancreatic Rat Islets via Adenoviral-Induced Expression of Glycerol Kinase Is Mediated via Mitochondrial Metabolism

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In this study, we examined whether adenoviral-mediated glycerol kinase (AdV-CMV-GlyK) expression in isolated rat pancreatic islets could introduce glycerol-induced proinsulin biosynthesis. In AdV-CMV-GlyK–infected islets, specific glycerol-induced proinsulin biosynthesis translation and insulin secretion were observed in parallel from the same islets. The threshold concentration of glycerol required to stimulate proinsulin biosynthesis was lower (0.25–0.5 mmol/l) than that for insulin secretion (1.0–1.5 mmol/l), reminiscent of threshold differences for glucose-stimulated proinsulin biosynthesis versus insulin secretion. The dose-dependent glycerol-induced proinsulin biosynthesis correlated with the rate of glycerol oxidation in AdV-CMV-GlyK–infected islets, indicating that glycerol metabolism was required for the response. However, glycerol did not significantly increase lactate output from AdV-CMV-GlyK–infected islets, but the dihydroxyacetone phosphate (DHAP) to α-glycerophosphate (α-GP) ratio significantly increased in AdV-CMV-GlyK–infected islets incubated at 2 mmol/l glycerol compared with that at a basal level of 2.8 mmol/l glucose ($P \leq 0.05$). The DHAP:α-GP ratio was unaffected in AdV-CMV-GlyK–infected islets incubated at 2 mmol/l glycerol in the added presence of α-cyanohydroxycinnaminic acid (α-CHC), an inhibitor of the plasma membrane and mitochondrial lactate/pyruvate transporter. However, α-CHC inhibited glycerol-induced proinsulin biosynthesis and insulin secretion in AdV-CMV-GlyK–infected islets (>75%; $P = 0.05$), similarly to glucose-induced proinsulin biosynthesis and insulin secretion in AdV-CMV-GlyK–infected and control islets. These data indicated that in AdV-CMV-GlyK–infected islets, the importance of mitochondrial metabolism of glycerol was required to generate stimulus–response coupling signals to induce proinsulin biosynthesis and insulin secretion. *Diabetes* 50:1791–1798, 2001

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AdV-CMV-GlyK, adenoviral-mediated glycerol kinase; AdV-CMV-βGal, adenoviral-mediated β-galactosidase; α-CHC, α-cyano-4-hydroxycyanocinnamate; α-GP, α-glycerophosphate (glycerol 3-phosphate); BSA, bovine serum albumin; DHAP, dihydroxyacetone phosphate.

Circulating glucose concentration is the most physiologically relevant influence on the regulation of proinsulin biosynthesis and insulin secretion in pancreatic β-cells (1). Indeed, the regulation of proinsulin biosynthesis and the regulation of insulin secretion are tightly coupled, so that under normal circumstances intracellular insulin stores are maintained optimally to give a readily available pool for a rapid insulin secretion upon demand (1–3). The proximal metabolic and ion-channel events for the stimulus–response coupling mechanism of nutrient-induced insulin secretion in pancreatic β-cells have been relatively well defined (4–6). It has been suggested that an increase in β-cell glycolysis leads to a subsequent increase in cytosolic NADH production and that transfer of reducing equivalents from the cytosol to the mitochondria via shuttle mechanisms and subsequent entrance into the electron transport chain will lead to increased ATP production, bypassing a need for anaplerosis (5,7,8). However, a major role of the glycerol-phosphate shuttle in metabolic stimulus coupling for insulin release has been recently questioned (9,10). Indeed, there is accumulating evidence to indicate the importance of anaplerosis in generating secondary stimulus-coupling signals for nutrient-stimulated insulin release (4,11–14). Notwithstanding, glycerol-phosphate shuttle activity and anaplerosis should be linked intrinsically, as the shuttle is involved in replenishing β-cell cytosolic NAD^+ levels necessary to maintain glycolysis, which in turn provides pyruvate for anaplerosis (4,11,12).

In the short term (<2 h), glucose-induced proinsulin biosynthesis is regulated predominately at the translational level (2,15–18). However, unlike that for nutrient-induced insulin secretion, the stimulus–response coupling mechanism that specifically regulates proinsulin biosynthesis is poorly defined. In general, nutrient fuels that stimulate insulin secretion also specifically increase the rate of proinsulin biosynthesis above that of general protein synthesis in the β-cell (2). This suggests that the mechanisms of nutrient-induced proinsulin biosynthesis and insulin secretion share common metabolic requirements in the proximal part of the stimulus-response coupling pathways. However, considering that the downstream targets for regulation of proinsulin biosynthesis (i.e., translational machinery for protein synthesis) and insulin se-

cretion (i.e., β-granule transport and exocytosis) are distinct, there must be a point of divergence in the respective metabolic stimulus-response coupling pathways (2,12). Indeed, there is variance in the characteristics of glucose-induced biosynthesis versus insulin secretion that indicates differences in the metabolic regulation of these important β-cell functions. For example, glucose metabolism is required for glucose-induced proinsulin biosynthesis (1,12,17), but the threshold glucose concentration (2–4 mmol/l) is less than that required for glucose-induced insulin secretion (4–6 mmol/l) (1,2). Also, fatty acids markedly potentiate glucose-induced insulin secretion but have a modest inhibitory effect on glucose-induced proinsulin biosynthesis at the translational level (12,19). This suggests that cytosolic long-chain acyl-CoA is unlikely to play a signaling role in nutrient regulation of proinsulin biosynthesis in β-cells and that a divergence point in the metabolic signaling pathways for proinsulin biosynthesis and insulin secretion lies upstream of an elevation in cytosolic long-chain acyl-CoA (12).

Glycerol normally is not metabolized in pancreatic β-cells (20). However, it has been shown that adenovirally mediated expression of glycerol-kinase activity in pancreatic β-cells rendered significant glycerol-induced insulin secretion (20). In this study, it is demonstrated that adenoviral-mediated expression of glycerol-kinase activity in pancreatic islet cells caused a specific glycerol-induced proinsulin biosynthesis at the translational level in primary β-cells. Further characterization of glycerol-stimulated proinsulin biosynthesis and insulin secretion from glycerol-kinase–expressing islets suggested that glycerol was entering the lower part of glycolysis and providing downstream glycolytic metabolites for increased anaplerosis, as well as raising glycerol-phosphate shuttle activity. This indicated the importance of mitochondrial metabolism and anaplerosis for generating downstream signals important for metabolic regulation of pancreatic β-cell function (11,12).

RESEARCH DESIGN AND METHODS

Materials. L-[^{35}S]methionine (1,200 Ci/mmol), D-[U-^{14}C]glucose (250–360 mCi/mmol), and [U-^{14}C]glycerol (125–180 mCi/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). [^{125}I]-insulin (374 mCi/mg) was a gift from Eli Lilly (Indianapolis, IN). Pansorbin was purchased from Calbiochem (La Jolla, CA). Rat insulin was measured by radioimmunoassay using a kit from Linco (St. Louis, MO). Unless otherwise stated, all other chemicals were purchased from Sigma Chemical or Fisher Scientific (Fair Lawn, NJ) and were of the highest grade and purity available.

Rat pancreatic islet isolation and recombinant adenovirus infection. Pancreatic islets of Langerhans were isolated from male 150-g Sprague-Dawley rats by collagenase digestion and Histopaque-Ficoll density gradient centrifugation as described previously (21). Batches of 800 isolated islets were placed in 60-mm diameter Petri dishes in 5 ml of RPMI-1640 medium containing 11 mmol/l glucose supplemented with 10% (vol/vol) fetal calf serum, 200 units/ml penicillin, and 200 mg/ml streptomycin for 1–2 h at 37°C in 95% air/5% CO_2. Purified replication-deficient recombinant adenovirus capable of expressing *Escherichia coli* glycerol kinase (AdV-CMV-GlyK) or β-galactosidase (AdV-CMV-βGal) in primary cells was prepared as described previously (20). First, 10 μl of AdV-CMV-GlyK or AdV-CMV-βGal stock virus preparations (1×10^{13} pfu/ml) was added to a batch of 800 isolated islets and incubated for 1 h at 37°C. The medium was then replaced with fresh RMPI-1640 medium, and the islets were incubated for an additional 16 h at 37°C. This procedure generated a 25-fold multiplicity of infection, equivalent to that used in previous studies for AdV-CMV-GlyK–mediated glycerol-kinase expression (20). After islets had been infected with AdV-CMV-GlyK or AdV-CMV-βGal and subsequently cultured for 16 h, they were collected and assessed for proinsulin biosynthesis and insulin secretion as described below.

Measurement of glycerol-kinase activity. Glycerol-kinase activity was measured essentially as previously described (20,22). Briefly, 100 AdV-CMV-GlyK– or AdV-CMV-βGal–infected pancreatic islets were suspended in an extraction buffer consisting of 50 mmol/l HEPES, 40 mmol/l KCl, 11 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, and 1% Triton X-100 (pH 7.8) and frozen in liquid nitrogen. Islet-cell suspensions were thawed on ice and sonicated (25 W, 10 s), and cell debris was pelleted by centrifugation at 20,000*g* for 15 min at 4°C. Samples of islet extract supernatant (10 μg of protein) was added to a 50-μl total volume assay buffer containing 100 mmol/l Tris/HCl (pH 7.2), 5 mmol/l ATP, 10 mmol/l $MgCl_2$, 100 mmol/l KCl, 2.5 mmol/l dithiothreitol, 4 mmol/l glycerol, and [U-^{14}C]glycerol (10 μCi/ml) and incubated for 2 h at 25°C. The reaction was terminated by the addition of 100 μl of 97% ethanol, 3% methanol. Fifty microliters of the terminated reaction mix was spotted onto circular DEAE filters (Schleieicher & Schuell, Keene, NH) and air-dried using a filtration manifold (Millipore, Bedford, MA). The filters then were washed five times with 100 ml of distilled water and then air-dried. Dried filters were placed in scintillation vials and counted in 10 ml of BioSafe II scintillation mixture (Research Products International, Mount Prospect, IL).

Parallel analysis of proinsulin biosynthesis and insulin secretion in isolated rat islets. Essentially, a method previously described for MIN6 cells was followed (12). For each observation, ~50 AdV-CMV-GlyK– or AdV-CMV-βGal–infected islets were preincubated in 1.5-ml microcentrifuge tubes in 300 μl of Krebs-Ringer bicarbonate buffer, 16 mmol/l HEPES, and 0.1% (wt/vol) bovine serum albumin (BSA) containing 2.8 mmol/l glucose for 1 h at 37°C. Cells were centrifuged at 800*g* for 2 min, resuspended, then incubated for 1 h in 300 μl of the same buffer containing insulin secretagogues as indicated. The last 20 min of this incubation period was in the presence of 100 μCi/ml [^{35}S]methionine to monitor protein biosynthesis (12,18,21). For assessment of insulin secretion in the same cells, a 200-μl aliquot of this second incubation medium was collected and centrifuged at 1,500*g* for 2 min to remove debris, and the supernatant was saved and stored at −80°C pending analysis of insulin secreted by radioimmunoassay. The islets then were washed in 500 μl of phosphate-buffered saline containing 0.1% (wt/vol) BSA, then resuspended in 300 μl of lysis buffer and lysed as described previously (12,18,21). A 5-μl aliquot of the resulting lysate was stored at −80°C pending analysis of intracellular insulin content by radioimmunoassay. Another 5-μl aliquot of the lysate was taken for assessment of total protein synthesis by trichloroacetic acid (TCA) precipitation as described previously (18,21). Subsequently, all cell lysates were equilibrated for equivalent levels of total protein synthesis before analysis of proinsulin biosynthesis so that specific regulation of proinsulin synthesis above that of general protein synthesis in the β-cell could be assessed (18,21). The remaining equilibrated cell lysate was subjected to specific (pro)insulin immunoprecipitation for assessment of proinsulin biosynthesis, with analysis by alkaline-gel electrophoresis, fluorography, and densitometric scanning as described (18,21).

Measurement of α-glycerophosphate and dihydroxyacetone. The ratio of α-glycerophosphate (α-GP) to dihydroxyacetone phosphate (DHAP) was assessed as described previously (12). Briefly, groups of 500 AdV-CMV-GlyK– or AdV-CMV-βGal–infected rat islets were incubated for 1 h at 37°C in 300 μl of Krebs-Ringer bicarbonate buffer, 16 mmol/l HEPES, and 0.1% (wt/vol) BSA containing 2.8 mmol/l glucose, 16.7 mmol/l glucose, or 5 mmol/l glycerol as indicated. At the end of this incubation period, the islets were centrifuged at 500*g* for 3 min, and the supernatant was removed and stored at −80°C pending radioimmunoassay for insulin secretion. The islets were washed in phosphate-buffered saline, then resuspended in 400 μl of ice-cold 5 mmol/l HEPES-NaOH buffer (pH 7.2) containing 60 mmol/l sucrose, 190 mmol/l mannitol, 15 mmol/l KCl, 3 mmol/l KH_2PO_4, 1 mmol/l $MgCl_2$, and 0.5 mmol/l EDTA and homogenized on ice using 25 strokes of a Potter homogenizer. The homogenate then was centrifuged (1,500*g*, 10 min, 4°C) to remove nuclear and whole cells. The supernatant was removed, and the resultant pellet was resuspended in 400 μl of the same buffer and rehomogenized as above. The two resultant supernatants were combined. A 10-μl aliquot of the homogenate was stored at −80°C pending analysis of protein content, and the remaining homogenate was deproteinized with 6N $HClO_4$ and stored at −80°C. Before analysis, the samples were neutralized to pH 7.2 with 5 mol/l K_2CO_3, and the protein precipitate was removed by centrifugation at 10,000*g* for 10 min at 4°C. The α-GP and DHAP levels were measured by fluorometric enzyme assays as previously outlined (12,23).

Measurement of lactate output. Groups of either 200 AdV-CMV-GlyK– or AdV-CMV-βGal–infected rat islets were incubated for 1 h at 37°C in 300 μl of Krebs-Ringer bicarbonate buffer, 16 mmol/l HEPES, and 0.1% (wt/vol) BSA containing 2.8 mmol/l glucose, 16.7 mmol/l glucose, or 0.1–5.0 mmol/l glycerol as indicated. The islets then were centrifuged at 500*g* for 3 min, and a 150-μl aliquot of the incubation medium was removed for analysis of lactate output as previously described (12,20,24). The islets were resuspended in lysis buffer and analyzed for protein content as outlined (12,18,21).

Measurement of glucose and glycerol oxidation. Glycerol and glucose oxidation assays were performed as previously described (25,26). Briefly, groups of 25–30 islets were suspended in 200 μl of Krebs-Ringer bicarbonate buffer, 16 mmol/l HEPES, and 0.1% (wt/vol) BSA containing 2.8 mmol/l glucose, 16.7 mmol/l glucose, or 0.1–5.0 mmol/l glycerol in rounded bottom cups sealed with a rubber-sleeved stopper. Then, $\sim 2 \times 10^5$ cpm of [U-^{14}C]glucose in samples containing glucose as a substrate or [U-^{14}C]glycerol in samples containing glycerol as a substrate were added to the islet suspensions. The cups were then sealed within a 20-ml borosilicate glass scintillation vial using a rubber-sleeved stopper and incubated for 2 h at 37°C. Then islet-cell oxidation was halted by the addition of 20 μl of sodium phosphate buffer (pH 6.0) containing 50 mmol/l rotenone to the islets in the cups via the sleeved stoppers. Afterward, 100 μl of $HClO_4$ was added to the islets in the cup and 300 μl of 1 mol/l benzethonium was added to the bottom of the scintillation vials via the appropriate sleeved stoppers. The samples then were incubated for an additional 2 h at 37°C in a shaking water bath. Then the seals were removed and the cups were discarded, and 10 ml of BioSafe II scintillation cocktail was added to the vials. The vials then were kept at 25°C overnight before scintillation counting.

Other methods. Protein was assayed by the bicinchoninic acid method (Pierce, Rockford, IL). Data are presented as means ± SE. Statistical significance between groups was analyzed using Student's t test; $P \leq 0.05$ was considered significant.

RESULTS

Recombinant adenovirus-mediated expression of glycerol-kinase activity in rat pancreatic islets. Recombinant adenovirus-mediated gene transfer to isolated rat pancreatic islets in vitro gave a >80% efficiency of gene transfer to the islet cells, similar to that previously described (27,28). Infection of isolated rat islets with AdV-CMV-GlyK gave a glycerol-kinase activity of 360 ± 42 nmol glycerol 3-phosphate/mg protein per h ($n = 5$) at 16 h after infection, comparable with that previously observed in INS-1 cells (20). Little glycerol-kinase activity (<1.5 nmol glycerol 3-phosphate/mg protein per h) was found in either uninfected or AdV-CMV-βGal–infected controls at 16 h after infection. This was supportive of a recent study in which it was demonstrated that there is negligible glycerol-kinase activity in either isolated rat pancreatic islets or pancreatic β-cell line INS-1, unless glycerol-kinase protein expression is introduced via AdV-CMV-GlyK (20).

Recombinant adenovirus-mediated expression of glycerol-kinase–introduced glycerol oxidation, glycerol-induced proinsulin biosynthesis, and insulin secretion in rat pancreatic islets. In control AdV-CMV-βGal–infected pancreatic islets, glycerol did not induce an increase in proinsulin biosynthesis (Fig. 1*A*) or insulin secretion (Fig. 1*B*), and AdV-CMV-βGal–infected islets oxidized glycerol minimally (Fig. 1*C*). Similar results were found in uninfected control islets (data not shown). However, in AdV-CMV-GlyK–infected islets, glycerol specifically stimulated proinsulin biosynthesis above that of total protein synthesis (Fig. 1*A*), and there was significant glycerol-induced insulin secretion (Fig. 1*B*). Moreover, AdV-CMV-GlyK–infected islets demonstrated a dose-dependent increase in glycerol oxidation that was not apparent in AdV-CMV-βGal–infected control islets (Fig. 1*C*). Glycerol-stimulated proinsulin biosynthesis and insulin secretion in AdV-CMV-GlyK–infected islets was independent of the presence of glucose (Fig. 1*A* and *B*). The threshold concentration of glycerol required to specifically increase the rate of proinsulin biosynthesis above that of total protein synthesis in AdV-CMV-GlyK–infected islets was between 0.25 and 0.5 mmol/l glycerol (Fig. 1*A*). Half-maximal stimulation of proinsulin biosynthesis was achieved at 0.6 ± 0.1 mmol/l glycerol ($n = 5$) that reached a maximum stimulation at 1 mmol/l glycerol, sixfold ($P \leq 0.002$) above that at basal 0.1 mmol/l glycerol or 2.8 mmol/l glucose (Fig. 1*A*). Maximum stimulation of proinsulin biosynthesis at >1 mmol/l glycerol was not significantly different from that at 16.7 mmol/l glucose in AdV-CMV-GlyK– or AdV-CMV-βGal–infected islets (Fig. 1*A*). Glycerol-induced proinsulin biosynthesis was most likely occurring at the translational level as established previously for a 1-h stimulatory period (12,15,16,18,21). Preproinsulin mRNA levels remained the same in AdV-CMV-GlyK–infected islets incubated for 1 h at either 0.25 mmol/l or 2 mmol/l glycerol and likewise at 2.8 mmol/l or 16.7 mmol/l glucose (data not shown). Moreover, an inhibitor of transcription, actinomycin-D (10 μg/ml) (17,21), had no effect on glycerol-stimulated proinsulin biosynthesis (data not shown), as observed previously for glucose-induced proinsulin biosynthesis (12,17,18,21).

The threshold concentration of glycerol required to stimulate insulin secretion in AdV-CMV-GlyK–infected islets was higher than that required for glycerol-stimulated proinsulin biosynthesis: between 1.0 and 1.5 mmol/l glycerol (Fig. 1*B*). Half-maximal stimulation of insulin secretion in AdV-CMV-GlyK–infected islets was achieved at 1.7 ± 0.2 mmol/l glycerol ($n = 5$), which reached a maximum stimulation at 5 mmol/l glycerol, fourfold ($P \leq 0.01$) above that at a basal 0.1 mmol/l glycerol or 2.8 mmol/l glucose (Fig. 1*B*). A similar pattern of glycerol-induced insulin secretion has been observed in AdV-CMV-GlyK–infected INS-1 cells (20).

The threshold concentration of glycerol required to gain significant glycerol oxidation in AdV-CMV-GlyK–infected islets was between 0.25 and 0.5 mmol/l glycerol (Fig. 1*C*), similar to that required to initiate glycerol-stimulated proinsulin biosynthesis (Fig. 1*A*). A maximum rate of glycerol oxidation in AdV-CMV-GlyK–infected islets was achieved above 5 mmol/l glycerol, which was 12-fold higher ($P \leq 0.001$) than that at basal 0.25 mmol/l glycerol and 4-fold ($P = 0.005$) above basal glucose oxidation at 2.8 mmol/l glucose (Fig. 1*C*). Half-maximal glycerol oxidation was observed at 1.4 ± 0.2 mmol/l glycerol ($n = 3$; Fig. 1*C*).

Glycerol did not affect lactate output in glycerol-kinase–expressing rat pancreatic islets. In both AdV-CMV-GlyK– and AdV-CMV-βGal–infected islets was a 2.5-fold increase ($P = 0.02$) in lactate output at a stimulatory 16.7 mmol/l glucose compared with that at a basal 2.8 mmol/l glucose (Fig. 2). In contrast, between 0.2 and 2 mmol/l glycerol, there was no significant change in lactate output from either AdV-CMV-GlyK– or AdV-CMV-βGal–infected islets compared with that at a basal 2.8 mmol/l glucose or 0.2 mmol/l glycerol (Fig. 2). At 5 mmol/l glycerol, a slight increase was observed in lactate output in AdV-CMV-GlyK–infected islets, compared with the basal rate in AdV-CMV-βGal–infected control islets; however, this was not statistically significant (Fig. 2). In the added presence of 1 mmol/l α-cyanohydroxycinnaminic acid (α-CHC), an inhibitor of the plasma membrane and mitochondrial lactate/pyruvate transporter (12,29), lactate output generally was reduced by ~70% ($P = 0.05$) in both AdV-CMV-GlyK– and AdV-CMV-βGal–infected islets whether incubated at 0.2–5 mmol/l glycerol or at 2.8 mmol/l or 16.7

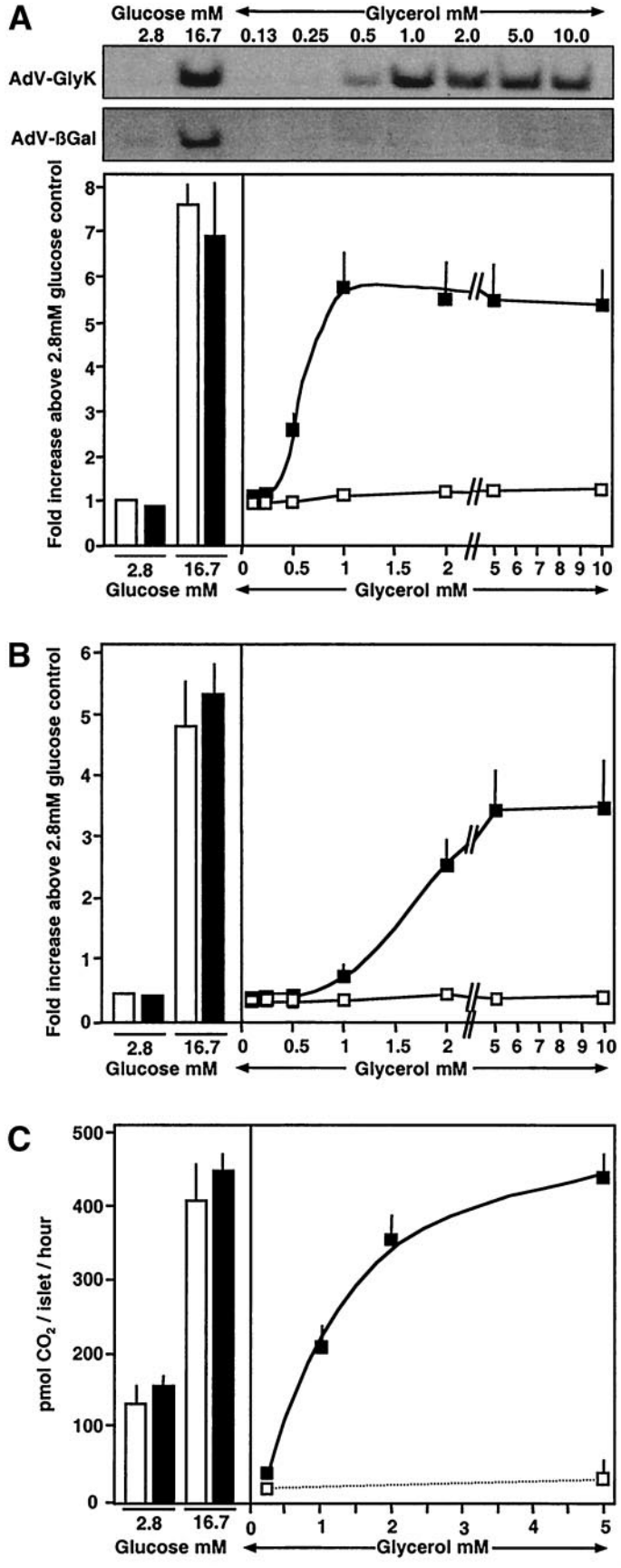

FIG. 1. Specific glycerol-stimulated proinsulin biosynthesis, insulin secretion, and glycerol oxidation in AdV-CMV-GlyK–treated isolated rat pancreatic islets. Isolated rat pancreatic islets were treated with either AdV-CMV-GlyK to express glycerol kinase or AdV-CMV-βGal to express β-galactosidase as a control and then incubated for 60 min at 2.8 mmol/l or 16.7 mmol/l glucose or at 0.13–10.0 mmol/l glycerol as indicated. Proinsulin biosynthesis was determined by immunoprecipitation, electrophoresis, fluorography, and densitometric scanning; insulin secretion was determined by radioimmunoassay; and glycerol oxidation was determined as described in RESEARCH DESIGN AND METHODS. *A*: Results for proinsulin biosynthesis in either AdV-CMV-GlyK–infected (■) or AdV-CMV-βGal–infected (□) islets. Results are means ± SE of at least five individual experiments, and an example fluorograph is shown. The relative increase in scanning volume density of the band on the autoradiograph equivalent to [^{35}S]proinsulin above that at a basal 2.8 mmol/l glucose was calculated for each analysis as described (21). *B*: Results for insulin secretion of either AdV-CMV-GlyK–infected (■) or AdV-CMV-βGal–infected (□) islets (means ± SE of at least five individual experiments). *C*: Results for glycerol and glucose oxidation in either AdV-CMV-GlyK–infected (■) or AdV-CMV-βGal–infected (□) islets (means ± SE of at least four experiments).

mmol/l glucose, confirming α-CHC as an inhibitor of lactate export (data not shown).

Glycerol increases α-GP and DHAP levels in glycerol-kinase–expressing rat pancreatic islets. An indication of α-GP shuttle activity can be gained by measuring the intracellular levels of α-GP and DHAP, which has been proposed as being reflective of a cytosolic NAD^+/NADH ratio (30–32). In AdV-CMV-GlyK– and AdV-CMV-βGal–infected islets incubated at 16.7 mmol/l glucose, there was a significant increase in DHAP levels compared with that at a basal 2.8 mmol/l glucose ($P = 0.05$; Table 1). In contrast, α-GP levels did not change significantly in the same islets, so the DHAP:α-GP ratio increased significantly upon increasing the glucose concentration from 2.8 to 16.7 mmol/l glucose ($P = 0.05$; Table 1) as observed previously (12,32). In AdV-CMV-βGal–infected islets incubated at 2 mmol/l glycerol, the levels of DHAP and α-GP as well as the DHAP:α-GP ratio did not change significantly from AdV-CMV-GlyK– or AdV-CMV-βGal–infected islets incubated at 2.8 mmol/l glucose (Table 1). However, AdV-CMV-GlyK–infected islets that were incubated at 2 mmol/l glycerol levels of DHAP increased twofold over that in AdV-CMV-βGal–infected control islets incubated at 2 mmol/l glycerol levels ($P = 0.01$; Table 1). In addition, α-GP levels were increased by >50% in AdV-CMV-GlyK–infected islets versus AdV-CMV-βGal–infected control islets incubated at 2 mmol/l glycerol ($P = 0.05$; Table 1). It should be noted that DHAP and α-GP levels in AdV-CMV-GlyK–infected islets incubated at 2 mmol/l glycerol were higher than those in AdV-CMV-GlyK– or AdV-CMV-βGal–infected islets incubated at 2.8 or 16.7 mmol/l glucose (Table 1). Notwithstanding, the DHAP:α-GP ratio in AdV-CMV-GlyK–infected islets incubated at 2 mmol/l glycerol was significantly elevated compared with AdV-CMV-βGal–infected control islets incubated at 2 mmol/l glycerol ($P = 0.02$) (Table 1), to a similar extent as that observed in islets incubated at a stimulatory 16.7 mmol/l glucose (Table 1) (12). DHAP levels, α-GP levels, and the DHAP:α-GP ratio were not affected in AdV-CMV-GlyK– or AdV-CMV-βGal–infected islets incubated at 2 mmol/l glycerol in the added presence of 1 mmol/l α-CHC (Table 1).

Mitochondrial metabolism was required for glycerol-induced proinsulin biosynthesis and insulin secretion in glycerol-kinase–expressing rat pancreatic islets. Pyruvate entry into mammalian cells and mitochondria can be inhibited by α-CHC (12,29). In the presence of 1 mmol/l α-CHC, 16.7 mmol/l glucose-stimulated proinsulin biosynthesis was inhibited significantly in both AdV-CMV-GlyK– and AdV-CMV-βGal–infected islets by ~60% ($P =$

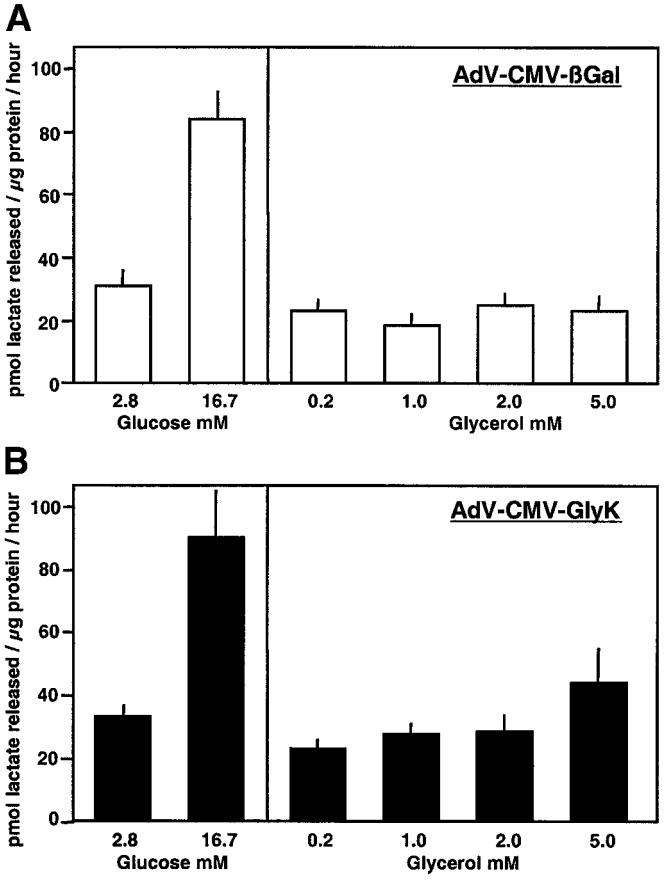

FIG. 2. Effect of glucose and glycerol on lactate output from AdV-CMV-GlyK–treated isolated rat pancreatic islets. Isolated rat pancreatic islets were treated with either AdV-CMV-βGal (*A*) to express β-galactosidase as a control or AdV-CMV-GlyK (*B*) to express glycerol kinase and then incubated for 60 min at 2.8 or 16.7 mmol/l glucose or at 0.2–5.0 mmol/l glycerol as indicated, and the media were analyzed for lactate output as described in RESEARCH DESIGN AND METHODS. Results from either AdV-CMV-βGal–infected (□) or AdV-CMV-GlyK–infected (■) islets are illustrated as means ± SE of at least four individual experiments.

0.01) (Fig. 3*A*). Glucose-induced insulin secretion also was inhibited significantly in AdV-CMV-GlyK– and AdV-CMV-βGal–infected islets by ~60% ($P = 0.005$) (Fig. 3*B*). In AdV-CMV-GlyK–infected islets, 2 mmol/l glycerol-induced proinsulin biosynthesis was inhibited significantly by ~75% in the presence of 1 mmol/l α-CHC ($P = 0.002$) (Fig. 3*A*), as was 2 mmol/l glycerol-induced insulin secretion ($P = 0.002$) (Fig. 3*B*). Glycerol did not induce proinsulin biosynthesis or insulin release in AdV-CMV-βGal–infected islets; 1 mmol/l α-CHC had no effect on the basal rate of proinsulin biosynthesis or insulin release observed at 2 mmol/l glycerol in these control islets (Fig. 3*B*). Finally, it was observed that the rate of glycerol oxidation at 2 mmol/l glycerol in AdV-CMV-GlyK–infected islets was inhibited significantly from 335 ± 54 pmol ($n = 3$) CO_2 produced from glycerol/islet/h to 87 ± 14 ($n = 3$) pmol CO_2/islet/h in the added presence of 1 mmol/l α-CHC ($P = 0.01$).

DISCUSSION

It has been shown that negligible glycerol-kinase activity is present in pancreatic β-cells, but recombinant adenovirus-mediated expression of glycerol kinase in pancreatic β-cells (INS-1) can "engineer" glycerol-stimulated insulin release (33). This study demonstrated that expression of glycerol kinase in primary isolated pancreatic rat islets can introduce not only glycerol-induced insulin release but also a parallel glycerol-induced increase of proinsulin biosynthesis at the translational level. This reaffirms the notion that the β-cell is poised exquisitely so that nutrients that stimulate insulin secretion also are able to increase specifically and rapidly the rate of proinsulin biosynthesis to maintain intracellular insulin stores at optimal levels (2,12).

Glycerol can be taken up by mammalian cells in proportion to the extracellular glycerol concentration (33,34). The expression of glycerol kinase at suitably high levels in pancreatic islet β-cells would generate α-GP from glycerol, which then could be converted to DHAP and enter the lower half of glycolysis, as DHAP is in equilibrium with glyceraldehyde-3-phosphate (33). This study found that the intracellular levels of DHAP and α-GP were markedly increased in AdV-CMV-GlyK–infected islets compared with AdV-CMV-βGal–infected control islets when incubated with glycerol, which resulted in a significant increase in the DHAP:α-GP ratio (similar to stimulatory 16.7 mmol/l glucose concentration [Table 1]). The oxidation of α-GP to DHAP could be achieved by a NAD^+-linked cytosolic glycerol phosphate dehydrogenase and/or via a mitochondrial FAD-linked glycerol phosphate dehydrogenase in the glycerophosphate shuttle. Formation of DHAP from α-GP by the cytosolic glycerol phosphate dehydrogenase is an equilibrium reaction that should not alter the DHAP:α-GP ratio (35). In contrast, oxidation of newly formed α-GP to DHAP by the FAD-linked glycerol phosphate dehydrogenase in the mitochondrial loop of the glycerophosphate shuttle does increase in the DHAP:α-GP ratio (5,14,20,35). Thus, because glycerol induced a significant increase in the DHAP:α-GP ratio in AdV-CMV-GlyK–infected islets, it was likely that a larger proportion of the DHAP was generated from glycerol (via α-GP) by the FAD-linked glycerol phosphate dehydrogenase in the mitochondrial loop of the glycerophosphate shuttle. A glycerophosphate shuttle–mediated increase in the DHAP:α-GP ratio would reflect an increase in the islet β-cell cytosolic NAD^+/NADH ratio, as well as provide a means for delivering reducing equivalents to the mitochondrial electron transport chain, at the FADH-linked complex-II, for subsequent generation of ATP (14,35). As such, increased NADH generated by increased glyceraldehyde-3-phosphate dehydrogenase activity as a consequence of increased DHAP glycolytic metabolism (5) could be compensated for efficiently by a coincidental increase in NAD^+ production from increased glycerophosphate shuttle activity so that glycolysis does not become hindered (4,5,36). Such NADH reoxidation in glycolysis is mediated by lactate dehydrogenase in mammalian cells, but primary pancreatic β-cells have very low lactate dehydrogenase activity, so NAD^+ production for glycolysis is mediated mostly through glycerophosphate shuttle activity (4,11,36). This is consistent with the fact that glycerol does not increase lactate output in AdV-CMV-GlyK–infected islets, as observed in this study. It was shown previously in the pancreatic β-cell line (INS-1) that AdV-CMV-GlyK–mediated expression of glycerol kinase could introduce glycerol-induced insulin secretion with a concomitant increase in lactate output (20).

TABLE 1
Glycerol-induced increase of α-GP and DHAP levels in AdV-CMV-GlyK–treated rat pancreatic islets

	2.8 mmol/l Glucose		16.7 mmol/l Glucose		2 mmol/l Glycerol		2 mmol/l Glycerol + α-CHC	
	AdV-CMV-βGal	AdV-CMV-GlyK	AdV-CMV-βGal	AdV-CMV-GlyK	AdV-CMV-βGal	AdV-CMV-GlyK	AdV-CMV-βGal	AdV-CMV-GlyK
DHAP (nmol/mg total protein)	39.1 ± 2.7	43.6 ± 5.8	77.8 ± 11.5	82.6 ± 8.4	40.4 ± 4.9	113.2 ± 6.3*	42.6 ± 5.4	103.2 ± 10.3*
α-GP (nmol/mg total protein)	30.7 ± 4.6	30.3 ± 5.1	36.0 ± 5.4	38.4 ± 4.3	28.0 ± 2.5	53.3 ± 4.6*	31.6 ± 3.5	48.1 ± 5.3*
DHAP:α-GP ratio	1.4 ± 0.1	1.5 ± 0.1	2.2 ± 0.5	2.2 ± 0.1	1.4 ± 0.1	2.1 ± 0.3*s	1.2 ± 0.1	2.2 ± 0.2*

Data are means ± SE for at least three independent determinations. Isolated rat pancreatic islets were treated with either AdV-GlyK to express glycerol kinase or AdV-CMV-βGal to express β-galactosidase as a control and then incubated for 60 min in 2.8 or 6.7 mmol/l glucose or in 2 mmol/l glycerol ± 1 mmol/l α-CHC as indicated. The concentrations of α-GP and DHAP were then measured in cell lysates as described in RESEARCH DESIGN AND METHODS. *Significant difference between AdV-CMV-GlyK–treated and AdV-CMV-βGal–treated islets (at least $P \leq 0.05$).

FIG. 3. Inhibition of mitochondrial pyruvate transport by α-CHC inhibits glycerol-stimulated proinsulin biosynthesis and insulin secretion in AdV-CMV-GlyK–treated isolated rat pancreatic islets. Isolated rat pancreatic islets were treated with either AdV-CMV-GlyK to express glycerol kinase or AdV-CMV-βGal to express β-galactosidase as a control and then incubated for 60 min at 2.8 or 16.7 mmol/l glucose or at 2 mmol/l glycerol ± 1 mmol/l α-CHC as indicated. Proinsulin biosynthesis was determined by immunoprecipitation, electrophoresis, logography, and densitometric scanning; and insulin secretion was determined by radioimmunoassay as described in RESEARCH DESIGN AND METHODS. *A*: Results for proinsulin biosynthesis in either AdV-CMV-GlyK–infected (■) or AdV-CMV-βGal–infected (□) islets. Results are means ± SE of at least three individual experiments, and an example logograph is shown. The relative increase in scanning volume density of the band on the autoradiograph equivalent to [^{35}S]proinsulin above that at a basal 2.8 mmol/l glucose was calculated for each analysis as described (21). *B*: Results for insulin secretion of either AdV-CMV-GlyK–infected (■) or AdV-CMV-βGal–infected (□) islets (means ± SE of at least three individual experiments).

However, insulinoma-derived β-cell lines contain high levels of lactate dehydrogenase activity (37,38), so cytosolic NAD^+ levels in INS-1 cells would likely be replenished via lactate production, lessening the need for increased glycerophosphate shuttle activity.

Notwithstanding, sufficient expression of glycerol kinase in pancreatic islets can generate meaningful levels of a glycolytic intermediate, DHAP, from glycerol for a nutrient fuel–induced stimulation of proinsulin biosynthesis and insulin secretion. It was interesting to note that the threshold concentration for glycerol-stimulated proinsulin biosynthesis was lower (0.25–0.5 mmol/l glycerol; Fig. 1*A*) as compared with that for stimulation of insulin secretion (1.0–1.5 mmol/l glycerol; Fig. 1*B*) in AdV-CMV-GlyK–infected islets. This was reflective of a lower threshold glucose concentration required to stimulate proinsulin biosynthesis translation (2–4 mmol/l glucose) versus insulin secretion (4–6 mmol/l glucose) (1), as well as that for pyruvate-stimulated proinsulin biosynthesis (0.13–0.2 mmol/l pyruvate) versus insulin secretion (0.2–0.3 mmol/l pyruvate) in MIN6 cells (12). It follows that the different threshold concentrations observed for glucose regulation of proinsulin biosynthesis versus insulin release do not necessarily lie at the level of glucose sensing, as proposed previously (1), but downstream in the generation of secondary metabolic stimulus-response coupling signals. Indeed, it was apparent that glycerol metabolism was required for a stimulus-response coupling of glycerol-induced proinsulin biosynthesis and insulin secretion in AdV-CMV-GlyK–infected pancreatic islets. The glycerol dose-response curve for glycerol-induced proinsulin biosynthesis paralleled that for glycerol oxidation in AdV-CMV-GlyK–infected islets, similar to that for glucose-induced proinsulin biosynthesis in normal pancreatic islets (1). Moreover, inhibition of glycerol oxidation by α-CHC similarly inhibited glycerol-induced proinsulin biosynthesis and insulin secretion in AdV-CMV-GlyK–infected islets to that for glucose-induced proinsulin biosynthesis and insulin secretion in control islets (Fig. 3). This suggested that in glycerol-stimulated AdV-CMV-GlyK–infected islets, the α-GP generated from glycerol kinase was being converted to DHAP (mostly via mitochondrial glycerol phosphate dehydrogenase, as discussed above) and entering the lower half of glycolysis to generate pyruvate. Pyruvate then can be either transported into mitochondria for entrance into the Krebs Cycle and subsequent oxidation or converted to lactate via lactate dehydrogenase, resulting in increased lactate output. However, as glycerol did not significantly increase lactate output from AdV-CMV-GlyK–infected islets, the pyruvate generated from glycerol was targeted preferentially for mitochondrial metabolism. This also is consistent with the relatively low lactate dehydrogenase activity and high pyruvate carboxylase activity in primary pancreatic islet β-cells (11,36). It also should be noted that addition of α-CHC did not affect a glycerol-induced increase in the DHAP:α-GP ratio in AdV-CMV-GlyK–infected islets, despite the α-CHC–mediated inhibition of glycerol oxidation and glycerol-induced proinsulin biosynthesis and insulin release. As such, it seemed unlikely that the glycerophosphate shuttle per se was generating downstream secondary signals to stimulated proinsulin biosynthesis and insulin release (4,12), although it cannot be ruled out that other mitochondrial shuttles could be playing a stimulus-coupling role in β-cells (5,8,13). However, other studies that used alternative experimental approaches have indicated that the glycerophosphate shuttle is not involved directly in generating stimulus-coupling signals for nutrient-stimulated insulin secretion in the β-cell (4,9,12).

In summary, this study indicated that in AdV-CMV-GlyK–infected primary β-cells, both glycerol-induced proinsulin biosynthesis and insulin release could be introduced in parallel, which were probably controlled from a secondary metabolic coupling signal(s) generated from mitochondria, via anaplerosis and/or glycerol oxidation, in agreement with previous studies that examined nutrient regulation of insulin release (4,11–14,38). It is not clear whether the same mitochondrial-derived metabolic stimulus-coupling signal controls both proinsulin biosynthesis translation and insulin secretion, and further experimentation is required. However, this study demonstrates further that the pancreatic β-cell has evolved a mechanism whereby nutrients coregulated proinsulin biosynthesis and insulin secretion, even when that nutrient is not normally metabolized unless engineered to do so by introduction of an enzyme that usually is not expressed. This provides a means to replenish insulin stores rapidly to optimal levels by the same stimulus that triggers insulin secretion.

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