A Pyruvate Cycling Pathway Involving Cytosolic NADP-dependent Isocitrate Dehydrogenase Regulates Glucose-stimulated Insulin Secretion

Glucose-stimulated insulin secretion (GSIS) from pancreatic islet β-cells is central to control of mammalian fuel homeostasis. Glucose metabolism mediates GSIS in part via ATP-regulated K⁺ (KATP) channels, but multiple lines of evidence suggest participation of other signals. Here we investigated the role of cytosolic NADP-dependent isocitrate dehydrogenase (ICDc) in control of GSIS in β-cells. Delivery of small interfering RNAs specific for ICDc caused impairment of GSIS in two independent robustly glucose-responsive rat isletoma (INS-1-derived) cell lines and in primary rat islets. Suppression of ICDc also attenuated the glucose-induced increments in pyruvate cycling activity and in NADPH levels, a predicted by-product of pyruvate cycling pathways, as well as the total cellular NADP(H) content. Metabolic profiling of eight organic acids in cell extracts revealed that suppression of ICDc caused increases in lactate production in both INS-1-derived cell lines and primary islets, consistent with the attenuation of pyruvate cycling, with no significant changes in other intermediates. Based on these studies, we propose that a pyruvate cycling pathway involving ICDc plays an important role in control of GSIS.

Glucose metabolism in pancreatic islet β-cells generates signals for acute stimulation of insulin secretion, and it is widely accepted that an increase in the ATP:ADP ratio brought about by glucose flux through glycolysis and the tricarboxylic acid cycle is central to this process. The rise in the ATP:ADP ratio results in closure of ATP-regulated K⁺ (KATP) channels, plasma membrane depolarization, activation of voltage-dependent Ca²⁺ channels, and subsequent influx of Ca²⁺ to stimulate insulin release (1, 2). This KATP channel-dependent pathway has been suggested to be especially important for the acute first phase of glucose-stimulated insulin secretion (GSIS). In the second and more prolonged phase of GSIS, glucose-derived factors in addition to ATP have been implicated (3, 4), including glutamate (5), malonyl-CoA/cytosolic long chain acyl-CoA esters (6–9), and transport of NADH reducing equivalents into the mitochondria via hydrogen shuttles (10), but data arguing against a role for some of these events have also been presented (11–13).

Pyruvate carboxylase (PC), which catalyzes the conversion of pyruvate to oxaloacetate, is highly active in β-cells and accounts for ~40–50% of pyruvate entry into mitochondrial metabolism at stimulatory glucose concentrations (14–17). The high PC activity in β-cells is remarkable in light of the absence of gluconeogenesis (18) and relatively low lipogenic activity (19) in these cells. Furthermore, it has been estimated that only 25% of the glucose-derived carbons entering the tricarboxylic acid cycle via PC are channeled into protein synthesis (17). These findings suggest that PC-catalyzed entry of metabolites into mitochondrial metabolic pathways (anaplerosis) may play other roles in β-cell function.

Previously, we examined glucose flux in a set of INS-derived cell lines with varying capacities for GSIS. These studies demonstrated a critical link between PC-mediated pyruvate exchange with tricarboxylic acid cycle intermediates (“pyruvate cycling”) and GSIS (15, 20). Moreover, in lipid-cultured cells, the normal glucose-induced increment in pyruvate cycling is diminished in concert with inhibition of GSIS (21). The 13C NMR-based isotopomer analysis used in these studies quantifies flux through the combined carboxylation (PC) and decarboxylation (malic enzyme (ME)) reactions associated with pyruvate cycling but does not precisely identify the pathways that contribute to this cycle. Pyruvate cycling can occur via the “pyruvate/malate cycle” involving PC-catalyzed conversion of pyruvate to oxaloacetate, reduction of oxaloacetate to malate, and subsequent conversion of malate to pyruvate by ME.

Glucose metabolism in pancreatic islet β-cells is remarkable in light of the absence of glucose-stimulated insulin secretion (GSIS) and its contribution to this cycle. The rise in the ATP:ADP ratio results in closure of ATP-regulated K⁺ (KATP) channels, plasma membrane depolarization, activation of voltage-dependent Ca²⁺ channels, and subsequent influx of Ca²⁺ to stimulate insulin release (1, 2). This KATP channel-dependent pathway has been suggested to be especially important for the acute first phase of glucose-stimulated insulin secretion (GSIS). In the second and more prolonged phase of GSIS, glucose-derived factors in addition to ATP have been implicated (3, 4), including glutamate (5), malonyl-CoA/cytosolic long chain acyl-CoA esters (6–9), and transport of NADH reducing equivalents into the mitochondria via hydrogen shuttles (10), but data arguing against a role for some of these events have also been presented (11–13).

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and decarboxylation of malate to pyruvate via the NADP-dependent cytosolic malic enzyme (MEc) or NAD-dependent mitochondrial malic enzyme (MEM). Alternatively, pyruvate cycling can take place via the “pyruvate/citrate” or “pyruvate/isocitrate” cycles, wherein the first and last steps are the same as in the pyruvate/malate cycle, but oxaloacetate formed in the PC reaction is converted to citrate or isocitrate, which then leave the mitochondria, after which malate is regenerated via cytosolic aconitase, citrate lyase, and malate dehydrogenase reactions. Furthermore, cytosolic isocitrate can be oxidized to α-ketoglutarate (αKG) via the cytosolic, NADP-dependent isocitrate dehydrogenase (ICDc), opening the possibility for a pyruvate cycling pathway involving re-entry of αKG into the mitochondria and reconversion to malate via enzymes of the tricarboxylic acid cycle. Interestingly, a predicted by-product of all of these pyruvate cycling pathways is NADPH, produced via MEc- or ICDe-catalyzed reactions.

In this study, we continue our investigation of the role of pyruvate cycling in the control of GSIS, and we specifically examine the potential involvement of ICDe in this process. We show that whole cell NADPH levels are closely correlated to insulin secretion when INS-1-derived 832/13 cells are stimulated with increasing concentrations of glucose. We also demonstrate that suppression of ICDe activity by adenovirus-mediated expression of ICDe-specific siRNAs strongly impairs GSIS in two INS-1-derived cell lines and primary rat islets, without affecting glycolytic flux or glucose oxidation in these cells. Moreover, suppression of ICDe impairs the glucose-induced increment in pyruvate cycling flux and NADPH levels and results in increases in lactate production in both INS-1-derived cell lines and primary rat islets, consistent with suppressed pyruvate cycling. Based on these observations, we propose that a pyruvate cycling pathway involving ICDe contributes to regulation of insulin secretion from pancreatic islets.

**EXPERIMENTAL PROCEDURES**

Reagents—All reagents and solutions were obtained from Sigma unless otherwise indicated.

Cell Lines—Two clonal cell lines, 832/3 and 832/13, were derived from parental INS-1-cells (22) via a transfection-selection strategy (23) and exhibit robust GSIS. Cells were cultured as described previously (23).

siRNA Duplex-mediated Gene Suppression—Expression of rat ICDe was suppressed by introducing siRNA duplexes (IDT, Coralville, IA) into 832/13 cells using the Amaxa nucleofection system (Amaxa Inc., Gaithersburg, MD) at a concentration of 2 μg per 3 million cells using program 20 with T solution. siRNA duplexes were targeted to 19-bp regions of the rat ICDe cDNA sequence (GenBank™ accession number NM_031510), beginning at nucleotides 290, 552, 600, 654, and 1100 relative to the start codon. A duplex with no known target (GAG ACC CTA TCC GTG ATT A) was used as control (siControl). Following nucleofection, cells were cultured for 72 h in 24-well plates prior to analysis of GSIS and ICDe mRNA levels.

Recombinant Adenovirus Construction—Adenoviruses containing siRNA sequences specific for ICDe (Ad-siICDe) or a sequence with no known gene homology as described above (Ad-siControl) were constructed using vectors EH006 and pJM17 as described recently (24, 25). The ICDe siRNA target sequence was GTA TGA TGG CAT CAA AGA (same sequence as the 654 siRNA duplex). Viruses were purified using a BD Biosciences Adeno-X purification kit (Clontech), and virus titer was calculated by end point dilution assay according to manufacturer’s guidelines.

**Adenovirus-mediated Gene Manipulation—**For cell fractionation, organic acid profiling, GSIS, NMR, and oxygen consumption studies, insulinoma cells were cultured in 15-cm dishes. For NADPH assays, cells were cultured in 6-well plates. For glucose usage, glucose oxidation, and glucose incorporation into fatty acids, cells were cultured in 12-well plates. After reaching 30% confluency, cells were transduced with a viral dose of 60 plaque-forming units/cell for 16 h and subsequently cultured for 72 h. The media were changed every 24 h.

Real Time PCR—RNA was isolated from islets and cells using the Qiagen RNeasy micro and mini kit (Qiagen Inc, Valencia, CA), respectively, and reverse-transcribed with the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s recommendations. Real time PCR analysis of ICDe mRNA levels was performed by using iTaq SYBR Green Supermix with ROX (Bio-Rad) and 900 nM of primers AAA ATA TCC CCC GGC TAG TGA (sense) and TCA TCA TTG GCC GAC ACG (antisense); prevalidated primers and probes against 18 S RNA were included as controls (Applied Biosystems, Foster City, CA). Measurements were performed on an ABI Prism 7000 sequence detection system.

Cell Fractionation and Immunoblot Analysis—Virus-treated cells were rinsed in cold PBS, scraped, and centrifuged at 2000 × g for 1 min at 4 °C. The cell pellet (~80 million cells) was resuspended in 1.0 ml of cold MSH buffer containing 220 mM mannitol, 70 mM sucrose, and 5 mM potassium/HEPES and homogenized in a Dounce homogenizer. Following a 10-min, 2700 × g centrifugation, the pellet was washed and rehomogenized in 400 μl of MSH. Supernatants from the homogenizations were pooled, and 50 μl was removed as a total cell lysate sample. Supernatants were centrifuged for 10 min at 12,000 × g. The supernatant and pellet fractions of this centrifugation step constituted the cytosolic and mitochondrial fractions, respectively. The total cell lysate, cytosolic, and mitochondrial fractions were resolved on a 10% BisTris NuPAGE NOVEX gel (Invitrogen) and transferred to polyvinylidene difluoride membranes (Invitrogen). Membranes were incubated with anti-ICD antibody (26) at a 1:1000 dilution for 1 h at room temperature, followed by secondary antibody treatment with horseradish peroxidase-conjugated anti-rabbit IgG at a 1:15,000 dilution. As a cytosolic fraction marker, the blots were reprobed with anti-GAPDH antibody (Abcam, Cambridge, UK) at a 1:4,000 dilution, followed by horseradish peroxidase-conjugated anti-mouse IgG at a 1:20,000 dilution. As a mitochondrial fraction marker, the blots were also treated with anti-cytochrome c oxidase subunit IV antibody (Mitosciences, Eugene, OR) at a 1:10,000 dilution, followed by horseradish peroxidase-conjugated anti-mouse IgG at a 1:30,000 dilution. Membranes were washed for at least 30 min in Tris-buffered saline, 0.1% Tween 20 (Bio-Rad) between antibody treatments.

**ICD Enzyme Activity**—ICD enzyme activity was measured in the total cell lysate, cytosolic, and mitochondrial fractions pre-
pared as described above. For each reaction, 10 μl of total cell lysate, mitochondrial, or cytosolic fractions was added to 180 μl of 100 mM Tris buffer, pH 8.0, containing 0.5 mM NADP, 3 mM MgCl₂, and 10% glycerol. ICD activity was measured spectrophotometrically by following NADPH formation at 340 nm, before and after addition of 1.5 mM isocitrate, in a total volume of 210 μl and normalized to protein concentration.

³¹C NMR Mass Isotopomer Analysis and GSIS—For GSIS assays, cells were preincubated in secretion buffer (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES, 2.5 mM CaCl₂, 0.2% bovine serum albumin, pH 7.2) containing 3 mM glucose for 1 h followed by incubation in secretion buffer containing 3 or 12 mM U-¹³C-labeled glucose (Cambridge Isotope Laboratories, Cambridge, MA) for 4 h. Samples of assay buffer were removed for insulin radioimmunoassay with the Coat-A-Count kit (DPC, Los Angeles, CA). Insulin content was measured as described previously (20). For NMR isotopomer analysis, cells were washed once with cold PBS and extracted with cold 3.5% perchloric acid. Extracts from three dishes per condition were pooled, neutralized, and lyophilized. The extract was dissolved in 0.5 ml of 2H₂O for measurement of pyruvate cycling activity relative to tricarboxylic acid cycle flux by ¹³C NMR as described previously (15, 20, 21).

Insulin Secretion in Primary Islets—Pancreatic islets were harvested from male Sprague-Dawley rats weighing ~250 g as described previously (27, 28). Following incubation for 1 h in RPMI 1640 medium containing 8 mM glucose and supplemented with 10% fetal bovine serum, 20 units/ml penicillin, 20 μg/ml streptomycin, 0.05 μg/ml amphotericin B (Invitrogen), islets were left untreated or transduced with either Ad-siControl or Ad-siICDc for 16 h at 2,000 plaque-forming units/islet. Islets were cultured for 72 h with media changes every 24 h, and GSIS was measured with 20 islets for each condition. Islets were preincubated in 500 μl of secretion buffer containing 2.8 mM glucose for 1 h and then incubated in 500 μl of secretion buffer containing either 2.8 mM glucose or 16.7 mM glucose for 2 h, with or without addition of 40 mM KCl. Islet DNA was harvested by addition of buffer containing 75% ethanol, 23.5% water, and 1.5% 6 N HCl. Samples were concentrated using a Savant SpeedVac SC110 (GMI, Ramsey, MN) and reconstituted with 10 mM Tris, 50 mM NaCl, 1 mM EDTA buffer, and DNA content was estimated by measurement absorbance of the solution at 260 nm.

Islet Lactate Assay—Islets treated with Ad-siControl or Ad-siICDc as described above were divided into groups of 30 and cultured in 500 μl of media containing 16.7 mM glucose for 16 h. For each reaction, 40 μl of media or of lactate standards (0–10 mM; Trinity Biotech, Bray, Ireland) from each group was combined with 200 μl of lactate assay reagent containing 175 mM hydrazine, 68 mM glycine, 11.3 mM NAD, and 2.9 mM EDTA. Lactate content was determined spectrophotometrically by measuring NADH formation at 340 nm, before and 1 h after addition of 10 μl of 500 units/ml lactate dehydrogenase.

NADPH Assay—NADP and NADPH were measured with a previously described procedure (29), with modifications (20).

Glycolytic Flux, Glucose Oxidation, and Glucose Incorporation into Lipids—832/13 cells were treated as described for NADPH studies, except that 5-³H-labeled glucose (Amersham Biosciences) was added to the secretion buffer at a specific activity of 0.02 Ci/mol to allow assay of glycolytic flux as described previously (30). For measurements of glucose oxidation, 832/13 cells were treated as described above, except that U-¹⁴C-labeled glucose (Amersham Biosciences) was included as tracer at a specific activity 0.5 Ci/mol as described previously (31). Cells from these plates were used for measurements of U-¹³C-labeled glucose incorporation into lipids. Cells were washed with 1 ml of PBS and processed by organic extraction as described previously (11). Oxygen consumption was measured as described previously (21).

Measurement of Organic Acids by Gas Chromatography/Mass Spectrometry (GC/MS)—For quantitative analysis of organic acids, cells were incubated for 2 h in secretion buffer containing 3 mM glucose followed by 2 h of incubation at either 3 or 12 mM glucose. Subsequently, cells were washed in 30 ml of ice-cold PBS, lysed on ice in 1 ml of ice-cold 0.1 M HCl, and subjected to GC/MS analysis as described previously (20).

Statistical Analysis—Data were expressed as the mean ± S.E. of at least three independent experiments performed in triplicate. Statistical significance was determined by two-sample equal variance Student’s t test for all assays. A p value of <0.05 was considered significant.

RESULTS

Cellular NADPH Levels Correlate with GSIS—An enzymatic cycling assay was employed to quantify NADPH and NADP in whole cell extracts. Cells were incubated with a range of 3–20 mM glucose, and insulin secretion, NADPH, and NADP was measured for each condition. Fig. 1 demonstrates that increases in glucose concentration over this range caused proportional increases in insulin secretion and NADPH:NADP ratio in 832/13 cells, consistent with a recent study in MIN6 cells and primary rat islets (32).

ICDc Suppression Impairs GSIS—Three genetically distinct ICD isoforms are expressed in pancreatic islet β-cells (3). First, β-cells contain the mitochondrial NAD-dependent ICD (EC 1.1.1.41), which participates in the tricarboxylic acid cycle. The additional two enzymes are NADP-dependent (EC 1.1.1.42) and are localized in cytosolic (ICDc) and mitochondrial compartments (ICDm), respectively. We treated 832/13 cells with...
five siRNA duplexes targeting ICDc mRNA, and we measured the extent of suppression of ICDc mRNA levels by real time PCR as well as the effect on GSIS. As shown in Fig. 2, duplexes 290 and 1100 decreased ICDc expression by 66 ± 6% and 28 ± 10%, respectively, and had no significant effect on insulin secretion at 12 mM glucose compared with cells treated with a control siRNA with no known target. In contrast, duplexes 552, 600, and 654 all decreased ICDc expression by more than 80%, and all significantly reduced insulin secretion at 12 mM glucose (p < 0.03). Because duplex 654 caused the most pronounced reduction in ICDc mRNA levels (92 ± 4%), this siRNA was cloned into an adenovirus (Ad-siICDc) for use in all subsequent experiments. Cells exposed to the nucleofection procedure in the absence of any siRNA demonstrated similar GSIS and ICDc RNA levels to cells treated with the control siRNA duplex (data not shown).

ICDc Is Effectively Silenced by a Recombinant Adenovirus Containing an ICDc-specific siRNA—As shown in Fig. 3A, cells treated with Ad-siICDc exhibited a 78 ± 5% reduction in ICDc mRNA compared with cells treated with Ad-siControl. ICDm mRNA levels were unchanged in Ad-siICDc-treated cells (data not shown).

The decrease in ICDc mRNA in response to Ad-siICDc treatment was accompanied by a 39 ± 9% decrease in NADP-dependent ICD activity in the cytosolic fraction and no significant change in activity in the mitochondrial fraction (Fig. 3B). Moreover, immunoblot analysis of fractionated cell lysates using an antibody that detects both ICDc and ICDm demonstrated 40 ± 14% and 60 ± 12% decreases in ICD immunoreactivity in the total extract and cytosolic fractions, respectively, with no change in ICD protein level in the mitochondrial fraction (Fig. 3C shows a representative blot from among four used for quantitative analysis). The relative enrichment of the cytosolic and mitochondrial fractions is indicated by the low levels of the mitochondrial protein cytochrome c oxidase IV and the cytosolic marker GAPDH in the cytosolic and mitochondrial compartments, respectively. Similar suppression of ICD mRNA, protein, and activity levels were observed in an independent robustly glucose responsive cell line, 832/3 (23, 25), in response to Ad-siICDc treatment (data not shown). In sum, these data demonstrate effective silencing of ICDc mRNA, protein, and enzyme activity in Ad-siICDc-treated cells. The lesser decrease in NADP-dependent ICD enzyme activity compared with ICDc mRNA or protein levels is probably because of some contaminating ICDm activity in the extract fractions.

siRNA Adenovirus-mediated Suppression of ICDc Impairs GSIS in INS-1-derived Cell Lines and Primary Rat Islets—Ad-siControl-treated 832/13 cells exhibited an 11.1 ± 2.1-fold increase in insulin secretion as glucose was raised from 3 to 12 mM. In contrast, Ad-siICDc treatment caused a 59 ± 7% decrease in insulin secretion at stimulatory glucose concentra-
Cytosolic Isocitrate Dehydrogenase and Insulin Secretion

We next investigated whether the effects of ICDc suppression affect non-fuel-mediated insulin release, we stimulated cells with a depolarizing concentration of K+ (40 mM). Addition of K+ caused an approximate 3-fold stimulation of insulin secretion relative to 2.8 mM glucose alone in both Ad-siControl- and Ad-siICDc-treated rat islets (Fig. 4B), indicating that suppression of ICDc does not interfere with nutrient-independent stimulation of insulin secretory granule exocytosis.

siRNA-mediated Suppression of ICDc Does Not Affect Glycolytic Flux or Glucose Oxidation but Alters Pyruvate Cycling—As expected, control cells exhibited significant increases in glycolytic flux (Fig. 5A) and glucose oxidation (Fig. 5B) as glucose was raised from 3 to 12 mM. Ad-siICDc treatment had no significant effect on glucose usage or oxidation at either glucose concentration (Fig. 5A and B) and also did not affect oxygen consumption (data not shown). Furthermore, Ad-siICDc treatment had no effect on cell viability, as measured by [3H]thymidine incorporation, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS), or adenylate kinase viability assays (data not shown). The MTS assay measures cell viability via metabolism of a tetrazolium compound in mitochondria and therefore indicates that no gross changes in mitochondrial function occurred as a consequence of Ad-siICDc treatment.

To determine whether ICDc influences pyruvate cycling, Ad-siICDc and Ad-siControl-treated 832/13 cells were incubated with 3 or 12 mM [U-13C]glucose, and pyruvate cycling was measured by 13C NMR mass isotopomer analysis (15). As expected, Ad-siControl-treated cells exhibited an increase in pyruvate cycling as glucose was raised from 3 to 12 mM (0.50 ± 0.08 to 1.07 ± 0.07; p < 0.001; Fig. 5C). Ad-siICDc treatment decreased pyruvate cycling at high glucose to 0.84 ± 0.04 (p < 0.02) and also caused a trend toward an increase at low glucose, such that the overall glucose-induced increment in pyruvate cycling decreased by 57% (from 0.57 ± 0.11 in Ad-siControl-treated cells to 0.25 ± 0.07 in Ad-siICDc-treated cells; p < 0.03) (Fig. 5D).

siRNA-mediated Suppression of ICDc Influences Cytosolic NADPH Levels—Given that NADPH is a by-product of the reaction catalyzed by ICDc, we next measured the impact of Ad-siICDc treatment on NADPH:NADP ratios and total nicotinamide adenine dinucleotide phosphate levels (NADPH and NADP). As shown in Fig. 6A, glucose caused an increase in NADPH and a decrease in NADP levels in both Ad-siICDc- and Ad-siControl-treated cells. The net effect is that the NADPH:NADP ratio tripled in response to high glucose in Ad-siControl-treated cells, increasing from 0.26 ± 0.02 to 0.78 ± 0.04 (p < 0.001; Fig. 6B). However, this glucose-induced rise in NADPH:NADP ratio was severely blunted in Ad-siICDc-treated cells, an effect due both to an increase at basal glucose (to 0.41 ± 0.03; p < 0.001) and a decrease at stimulatory glucose (to 0.64 ± 0.05; p = 0.05). Surprisingly, we also observed a small but significant decrease in the total nicotinamide adenine dinucleotide phosphate pool at high glucose in both control- (9.8 ± 1.6%; p < 0.002) and Ad-siICDc-treated cells (13.3 ± 1.9%; p < 0.016) compared with low glucose, with lower overall levels in the Ad-siICDc-treated cells (data not shown). Taken together,
these data demonstrate that Ad-siICDc treatment affects NADPH production at both low and high glucose.

Fatty acid synthesis and elongation are dependent on cytosolic NADPH. To test whether Ad-siICDc treatment affects cytosolic NADPH, 832/13 cells were treated with Ad-siICDc or Ad-siControl. A, NADPH and NADP levels were measured as described under “Experimental Procedures” in 832/13 cells exposed to 3 or 12 mM glucose. B, data of A expressed as the NADPH:NADP ratio. Results represent the mean ± S.E. for four independent experiments. C, [U-14C]glucose incorporation into lipids was measured at 3 and 12 mM glucose. Results represent the mean ± S.E. for three independent experiments. *, p < 0.05 relative to Ad-siControl.

Fatty acid synthesis and elongation are dependent on cytosolic NADPH. To test whether Ad-siICDc treatment affects cytosolic NADPH, 832/13 cells were incubated with [U-13C]glucose and extracted for NMR-based analysis of pyruvate cycling activity. Results represent the mean ± S.E. for five independent experiments. *, p < 0.05 relative to Ad-siControl. D, the data of C, plotted as the increment in pyruvate cycling as glucose, was raised from 3 to 12 mM. *, p < 0.03.
Cytosolic Isocitrate Dehydrogenase and Insulin Secretion

Effects of siRNA-mediated suppression of ICDc on organic acid levels—The levels of eight organic acids (lactate, pyruvate, fumarate, malate, citrate, isocitrate, succinate, and αKG) were measured in Ad-silICDc- and Ad-siControl-treated cells at basal and stimulatory glucose by quantitative GC/MS (Fig. 7A). Isocitrate was not detectable. In control cells, the levels of the other seven metabolites increased significantly as glucose was raised from 3 to 12 mM, as reported previously (20). There was no significant difference in levels of pyruvate, malate, citrate, succinate, or fumarate in Ad-silICDc- versus Ad-siControl-treated cells incubated with 12 mM glucose. In contrast, lactate levels were increased by 2.8-fold at 12 mM glucose in Ad-silICDc-treated versus control cells (p < 0.005), whereas αKG tended to decrease (4.7 ± 0.8 to 3.6 ± 0.6 nmol/mg protein; p = 0.14). Also, citrate levels were elevated at basal glucose (2.0 ± 0.6 to 3.9 ± 1.0 nmol/mg protein; p < 0.05) in Ad-silICDc-treated cells.

To determine whether Ad-silICDc also increased lactate levels in islets, secreted lactate was measured in media from isolated rat islets. As shown in Fig. 7B, lactate production increased from 4.5 ± 0.2 nmol/islet in Ad-siControl-treated islets to 6.0 ± 0.4 nmol per islet in Ad silICDc-treated islets (p < 0.005).

DISCUSSION

Pancreatic islet β-cells exhibit active anaplerosis, defined as entry of metabolites into mitochondrial pathways that leads to accumulation of tricarboxylic acid cycle intermediates. Sustained glucose stimulation in islet β-cells results in export of intermediates such as malate, citrate, isocitrate, and αKG from the mitochondria to the cytosol (33–35). Recent work has begun to provide evidence that this export of mitochondrial substrates is involved in the regulation of insulin secretion (3, 15, 20, 21, 32, 36, 37). For example, we previously demonstrated a strong correlation between PC-mediated pyruvate exchange with tricarboxylic acid cycle intermediates (pyruvate cycling) and GSIS in INS-1-derived cell lines with varying capacities for glucose response (15), and we also showed that lipid-induced impairment of GSIS results in ablation of the normal glucose-induced rise in pyruvate cycling activity (21). NADPH has been suggested as a coupling factor that may link pyruvate cycling activity and insulin secretion (3). NADPH produced in the cytosol through anaplerotic shuttle systems might be particularly important in β-cells because the pentose phosphate pathway, which produces substantial amounts of cytosolic NADPH in most cell types, is present at low activity in β-cells (16, 38, 39).

Interestingly, a recent study demonstrated a correlation between NADPH production and GSIS and also showed that increasing the NADPH:NADP ratio in pancreatic β-cells and MIN6 cells stimulated insulin granule fusion with the plasma membrane as measured by capacitance changes (32). However, studies investigating the effects of targeted manipulation of NADPH levels in intact β-cells have not yet been reported. Moreover, among the several different pathways by which pyruvate can be recycled in β-cells, the specific pathways involved in control of GSIS have not been identified.

In rat and human β-cells, NADPH is the by-product of both the MEc- and ICDc-catalyzed reactions, whereas in mouse, only the ICDc-mediated reaction contributes to NADPH generation because of the lack of MEc activity in mouse islets (40). Because ICDc is the universal participant in NADPH production in all three species, we focused the current studies on manipulation of ICDc expression in INS-1-derived (rat) cell lines and rat islets. The efficiency of our siRNA-based strategy for suppression of ICDc expression was demonstrated through reductions in ICDc mRNA, protein, and enzyme activity, with no significant effects on ICDm expression.

We found that suppression of ICDc in 832/13 cells resulted in both an increase in insulin secretion at basal glucose levels (3 mM glucose) and a decrease in insulin secretion at stimulatory glucose levels (12 mM glucose), such that the overall effect was to lower the glucose response from 11.1-fold in Ad-siControl-treated cells to 1.8-fold in Ad-silICDc-treated cells. Similar
results were obtained in a second independent INS-1-derived cell line, 832/3. Moreover, Ad-siICDc-mediated suppression of ICDc mRNA expression by 56 ± 6% in primary rat pancreatic islets lowered the glucose response from 8.1 ± 1.6 to 4.3 ± 0.9-fold, showing that the effects of ICDc suppression are recapitulated in primary cells and are not specific to insulinoma cells. The dramatic effect of Ad-siICDc treatment in these studies appears to be independent of glucose usage, glucose oxidation, or oxygen consumption, because all of these activities were unaffected by this manipulation in 832/13 cells. Instead, suppression of ICDc expression impacted pyruvate cycling activity in a manner analogous to its effects on insulin secretion. Thus, pyruvate cycling was significantly impaired at stimulatory glucose and showed a trend to increase at basal glucose levels.

$^{13}$C NMR-based isotopomer analysis quantifies flux through the combined carboxylation (PC) and decarboxylation (ME) reactions associated with pyruvate cycling but does not precisely identify the pathways that contribute to this cycle. Fig. 8 shows multiple pathways involving ICDc that may play an important role in pyruvate cycling (red arrows). Pyruvate entering the tricarboxylic acid cycle via PC can be converted to citrate and isocitrate, which exit the mitochondria via mitochondrial transporters. Cytosolic aconitase catalyzes conversion of citrate to isocitrate, and in turn, isocitrate can be converted to αKG via ICDc. Therefore, αKG may enter the mitochondria for conversion to malate by tricarboxylic acid cycle enzymes, and subsequent conversion to pyruvate by MEc or MEm, thus completing the pyruvate cycle.

FIGURE 8. Schematic diagram of pyruvate cycling pathways involving ICDc. Pyruvate cycling measured via $^{13}$C NMR isotopomer analysis can represent several cycling pathways. Potential pathways involving ICDc are represented as red arrows. Oxaloacetate, the product of PC-mediated entry of pyruvate into the tricarboxylic acid cycle, is converted to citrate and isocitrate. Both intermediates are capable of exiting the mitochondria via mitochondrial transporters. Cytosolic aconitase catalyzes conversion of citrate to isocitrate, and in turn, isocitrate can be converted to αKG via ICDc. Thereafter, αKG may enter the mitochondria for conversion to malate by tricarboxylic acid cycle enzymes, and subsequent conversion to pyruvate by MEm or MEc, thus completing the pyruvate cycle.

that the particular pyruvate cycling pathway in which ICDc is engaged provides an important link between glucose metabolism and insulin secretion. Interestingly, siRNA-mediated suppression of this cycling pathway does not impair insulin secretion in response to a non-nutrient stimulus such as K$^+$. This is consistent with the idea that pyruvate cycling makes its main contribution to the second amplification phase of insulin secretion, rather than the first triggering phase, because the latter is the phase that is thought to be regulated by ATP-sensitive K$^+$ channels (41, 42).

Ad-siICDc treatment caused a dramatic lowering of the NADP: NADPH ratio in response to stimulatory levels of glucose, combined with an increased ratio at basal glucose. These changes in the NADP: NADPH ratio correlated well with insulin secretion and pyruvate cycling. Also, at stimulatory glucose, there was a significant decrease in both NADPH and total NADP(H) content in Ad-siICDc-treated cells. Interestingly, it should be noted that another laboratory has also observed a decrease in total NADP(H) pools in response to glucose stimulation in MIN-6 cells and primary rat β-cells (32). NADPH has been reported to be co-secreted with insulin in toadfish islets (43), but the mechanism by which the total NADP(H) pool declines during glucose stimulation in mammalian islets and cell lines remains to be established.

Because the assays employed for measurement of NADPH and NADP levels did not discriminate between free and protein-bound nucleotides, or mitochondrial and cytosolic pools, we examined [U-$^{14}$C]glucose incorporation into lipids as an estimate of NADP(H) availability in the cytosol, because enzymes required for both fatty acid synthesis and elongation depend on cytosolic NADP(H). We observed a significant decrease in lipid synthesis in cells with decreased ICDc expression, consistent with the idea that the observed changes in total cellular NADP:H:NADPH ratio reflect changes in the cytosolic compartment. Taken together, these data are consistent with a link between ICDc expression, NADP(H) levels, and GSIS. However, it should be noted that our data could also support an important signaling role for cytosolic α-KG or a downstream metabolite of this intermediate, as has been suggested by a recent study (44). Further investigation of this point will be required.

If NADPH is an important factor, it has several functions that could relate to insulin secretion. First, it is a cofactor for numerous anabolic pathways, including fatty acid synthesis, desaturation, and elongation, all of which occur in β-cells (12, 45). In addition, NADPH is a substrate for 3-hydroxy-3-methylglutaryl-CoA reductase, an enzyme involved in the synthesis of
mevalonate, which is required for protein isoprenylation. Recently, isoprenylation has been suggested to be involved in GSIS (46). NADPH is also a cofactor for glutathione reductase and thioredoxin reductase, which play critical roles in protection against oxidative injury. Furthermore, iCDc has been implicated in the defense against oxidative stress-inducing reagents, such as γ-irradiation and oxidizing agents (47, 48). Thus, iCDc may play a role in maintaining a redox status in β-cells that is permissive for efficient insulin secretion, especially in islets subjected to stressful conditions, such as experienced during exposure to cytokines or high concentrations of fatty acids. Finally, NADPH has also been suggested to be a direct regulator of plasma membrane potential. In β-cells, voltage-dependent K⁺ (Kᵥ) channels repolarize the plasma membrane after glucose-mediated closure of KᵥATP channels (49). Kᵥ channels consist of integral membrane α-subunits, which form the pore, and regulatory β-subunits, which modify K⁺ channel gating. Interestingly, β-subunits containing NADPH oxidoreductase motifs have recently been identified in both human and rat islets (50), and direct binding of NADPH to NADPH oxidoreductase motifs have recently been identified in islets (51). Moreover, the rate of inactivation of these channels has been shown to be correlated with NADPH:NADP ratios (52), prolonging the repolarization phase of the plasma membrane current and possibly leading to enhanced insulin secretion.

In conclusion, our studies provide new evidence for a critical role of iCDc and pyruvate cycling in control of GSIS. Further studies will be required to understand the potential molecular links between NADPH or other metabolic by-products of the pyruvate/isocitrate cycle and insulin granule exocytosis. Meanwhile, these studies seem to contribute a clearer understanding of intra- and extramitochondrial metabolic pathways that regulate GSIS, information that may be useful for identification of targets that improve the performance of dysfunctional islets in type 2 diabetes.

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