

The Mitochondrial Citrate/Isocitrate Carrier Plays a Regulatory Role in Glucose-stimulated Insulin Secretion*

Received for publication, March 20, 2006, and in revised form, August 23, 2006. Published, JBC Papers in Press, September 25, 2006, DOI 10.1074/jbc.M602606200

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Glucose-stimulated insulin secretion (GSIS) is mediated in part by glucose metabolism-driven increases in ATP/ADP ratio, but by-products of mitochondrial glucose metabolism also play an important role. Here we investigate the role of the mitochondrial citrate/isocitrate carrier (CIC) in regulation of GSIS. Inhibition of CIC activity in INS-1-derived 832/13 cells or primary rat islets by the substrate analogue 1,2,3-benzenetricarboxylate (BTC) resulted in potent inhibition of GSIS, involving both first and second phase secretion. A recombinant adenovirus containing a CIC-specific siRNA (Ad-siCIC) dose-dependently reduced CIC expression in 832/13 cells and caused parallel inhibitory effects on citrate accumulation in the cytosol. Ad-siCIC treatment did not affect glucose utilization, glucose oxidation, or ATP/ADP ratio but did inhibit glucose incorporation into fatty acids and glucose-induced increases in NADPH/NADP⁺ ratio relative to cells treated with a control siRNA virus (Ad-siControl). Ad-siCIC also inhibited GSIS in 832/13 cells, whereas overexpression of CIC enhanced GSIS and raised cytosolic citrate levels. In normal rat islets, Ad-siCIC treatment also suppressed CIC mRNA levels and inhibited GSIS. We conclude that export of citrate and/or isocitrate from the mitochondria to the cytosol is an important step in control of GSIS.

The mechanism of glucose-stimulated insulin secretion (GSIS)² from pancreatic islet β -cells is not completely understood. One component of the signaling pathway involves glucose-induced increases in cytosolic ATP/ADP ratio, leading to

closure of K_{ATP} channels at the plasma membrane. K_{ATP} channel closure results in membrane depolarization and activation of voltage-dependent Ca²⁺ channels, increasing the concentration of cytosolic Ca²⁺ (1–4). Elevation of cytosolic Ca²⁺ promotes exocytosis of insulin-containing secretory granules (5). However, fluctuations in cytosolic Ca²⁺ are not the only signal, because under conditions of clamped cytosolic Ca²⁺ concentrations, glucose can still cause significant insulin secretion (6). This suggests that glucose generates signals/second messengers that are distinct from ATP and membrane depolarization for regulation of insulin secretion (7, 8). Some of the suggested mitochondrial factors include glutamate, malonyl-CoA, long-chain acyl-CoAs (LC-CoA), and/or NADPH (7–17).

The production of malonyl-CoA, LC-CoA, and NADPH in the cytosol depends on the export of mitochondrial metabolites. NADPH can be produced via one of three pyruvate cycling pathways, the pyruvate/malate pathway, the pyruvate/citrate pathway, or the pyruvate/isocitrate pathway, via cytosolic NADP⁺-dependent isoforms of malic enzyme (used in the pyruvate/malate and pyruvate/citrate pathways) or cytosolic, NADP⁺-dependent isocitrate dehydrogenase (ICDc) (used in the pyruvate/isocitrate cycle) (18, 19). Citrate emanating from mitochondrial metabolism can also be cleaved by ATP-citrate lyase to produce malonyl-CoA and LC-CoA (19). We and others have previously established that anaplerotic metabolism of pyruvate and pyruvate cycling flux are closely correlated with the capacity for glucose-stimulated insulin secretion in β -cells (9, 13, 18, 20, 21). Also supporting a key role for anaplerosis is the earlier finding that ~40–50% of pyruvate that enters mitochondrial pathways at high glucose does so via pyruvate carboxylase, the anaplerotic entry point (22–24).

Operation of pyruvate cycles and generation of stimulus/secretion coupling factors from these pathways requires efficient export of tri- and dicarboxylic acids from the mitochondria to the cytosol. Two proteins that mediate these activities are the dicarboxylate carrier, which primarily transports malate, and the tricarboxylate or citrate/isocitrate carrier (CIC), which catalyzes an electroneutral exchange of one of three tricarboxylic acids (citrate, isocitrate, or *cis*-aconitate) plus a proton, for another tricarboxylate-H⁺, a dicarboxylate (malate or succinate), or phosphoenolpyruvate (19). Mitochondrial CIC occupies a critical position in intermediary metabolism, serving as a key carbon source for the fatty acid and sterol biosynthetic

* This work was supported by a Canadian Institute of Health research fellowship (to J. W. J.), National Institutes of Health Grants DK42583 and DK58398 (to C. B. N.) and DK51610 (to C. J. R.), a grant from the Ministero dell'Istruzione, Università e Ricerca (to F. P.), and a sponsored research agreement with Takeda Pharmaceuticals (to C. B. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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² The abbreviations used are: GSIS, glucose-stimulated insulin secretion; CIC, citrate carrier; LC-CoA, long-chain acyl-CoA; ICDc, cytosolic isocitrate dehydrogenase; BTC, 1,2,3-benzenetricarboxylate; siRNA, small interfering RNA; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

pathways and for cytosolic NADPH production. Moreover, recent reports indicate that mouse islets lack malic enzyme activity (25) and that siRNA-mediated suppression of cytosolic NADP-dependent ICDC activity strongly impairs GSIS, pyruvate cycling, and NADPH production (18), suggesting that CIC could play a particularly important role in regulation of insulin secretion. In the current study, we have tested this hypothesis and demonstrate that inhibition of CIC with the specific substrate analogue 1,2,3-benzenetricarboxylate (BTC) or by siRNA-mediated suppression of its expression results in potent inhibition of GSIS, whereas CIC overexpression stimulates GSIS.

EXPERIMENTAL PROCEDURES

Cell Lines—The cell line 832/13 (26), derived from INS-1 rat insulinoma cells (27), was used in these studies. Cells were cultured, and insulin secretion assays were performed as previously described (26).

siRNA Duplexes and Construction of the Ad-siCIC and Ad-CIC Overexpression Recombinant Adenovirus—Four siRNA duplexes were tested against the rat citrate/isocitrate carrier (accession number L12016). Relative to the start codon, the first nucleotides targeted by each duplex were as follows: 136 (CIC 136; AGT CTT CAC GTA TTC GGT CTT), 233 (CIC 233; CCG AAT ACG TGA AGA CTC ATT), 682 (CIC 682; CCG TGA AGG TGA AAT TCA TTT), and 922 (CIC 922; GCT ACT GTA CTG AAG CAG GTT). A scrambled siRNA sequence with no known gene homology (GAG ACC CTA TCC GTG ATT A) was used as a control. siRNA duplexes were introduced into 832/13 cells at ~50% confluence by nucleofection, using the AMAXA system and the manufacturer's protocols (Gaithersburg, MD). Experiments were performed 3 days after duplex transfection.

The CIC136 and scrambled control siRNA sequences were used to prepare recombinant adenoviruses by previously described methods (28, 29). Recombinant adenoviruses containing the rat CIC cDNA sequence (AdCMV-CIC) or the bacterial β -galactosidase gene (AdCMV- β GAL) were prepared as previously described (30, 31). For both the siRNA and overexpression viruses, virus-containing medium was purified using a BD Biosciences Adeno-X Purification Kit (Clontech, Palo Alto, CA), and virus titer was estimated by measurement of absorbance at 260 nm.

Real Time PCR Analysis of CIC mRNA Expression—RNA was isolated from 832/13 cells using the Qiagen RNeasy Mini Kit and from primary rat islets using the Qiagen MicroRNA kit (Qiagen Inc., Valencia, CA). RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). CIC mRNA levels were detected by real time PCR as previously described (13, 18), using prevalidated CIC and 18 S RNA-specific fluorescent probes obtained from Applied Biosystems (Foster City, CA).

NADPH and NADP⁺ Assays—832/13 cells were pretreated for 2 h in 2.8 mM glucose and then incubated for 2 h at either 2.8 or 16.7 mM glucose. Cells were harvested and then snap frozen in dry ice/ethanol. The cell pellets were stored at -80°C until assayed. NADP⁺ and NADPH levels were measured as described (13, 15, 18).

Glucose Utilization—832/13 cells were cultured with [$5\text{-}^3\text{H}$]glucose as tracer at 0.08 Ci/mol, and samples were processed for measurement of glucose utilization as previously described (32).

Glucose Oxidation and Glucose Incorporation into Fatty Acids—832/13 cells were cultured with [$\text{U-}^{14}\text{C}$]glucose (0.5 Ci/mol) for 2 h, after which samples were loaded into a trap system containing 1 N NaOH loaded into adjacent wells. The trap system was closed, and then the wells with media were injected with 70% perchloric acid. The trap was incubated on a shaker at 125 rpm for 90 min. NaOH was transferred to scintillation vials containing UniScint BD scintillation fluid, mixed, and then counted. Glucose incorporation into lipid was measured as previously described (33).

ADP and ATP Determination—Cellular ATP and ADP content was determined at the end of the 2-h incubation period, as described (34, 35).

Mitochondrial Membrane Potential Measurements—Glucose-induced changes in mitochondrial membrane potential were quantified in 832/13 cells maintained at 37°C , as previously described (35). Cells were loaded with rhodamine 123 (2.6 μM Rh123 for 20 min) (Molecular Probes, Inc., Eugene, OR). Fluorescence was excited at 490 nm and measured at 530 nm. Images were captured and analyzed using Metamorph software (Molecular Devices Corp., Downingtown, PA). Exposure time was 0.2 s, and images were acquired at ~ 0.2 Hz. Cells were first treated with low glucose (2.8 mM) for 5 min and then with high glucose (20 mM) for 10 min. Changes in fluorescence were determined by comparison of the fluorescence at 20 mM glucose over the last 5 min of treatment with the average fluorescence over the first 50 s of exposure to 2.8 mM glucose, with the latter value set as 100%. At the end of an experiment, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added to assess cell viability and to test the ability of the cell loaded with Rh123 to respond to changes in the mitochondrial membrane potential induced by the chemical uncoupler.

Measurement of Citrate by Gas Chromatography/Mass Spectrometry—Total citrate levels were measured relative to an added $^2\text{H}_4$ -citrate internal standard (IsoTec) by gas chromatography/mass spectrometry, as previously described (13). Cytosolic and mitochondrial citrate levels were measured by the same method, with the following modifications. At the end of the secretion assay, cells were washed twice with phosphate-buffered saline and then treated with saponin (80 $\mu\text{g}/\text{ml}$) for 20 min (30 million cells/ml) to selectively permeabilize the plasma membrane of cells in suspension without causing cell death (36, 37). The cells were then centrifuged at $1000 \times g$ for 1 min. Supernatant was collected, representing the cytosolic fraction, and the cell pellet, representing the mitochondrial fraction, was resuspended in 650 μl of 0.1 N HCl, and each fraction was used for citrate analysis by gas chromatography/mass spectrometry.

CIC Immunoblot—Cellular proteins were extracted with cell lysis buffer (Cell Signaling) containing phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (10 $\mu\text{g}/\text{ml}$), aprotinin (10 $\mu\text{g}/\text{ml}$), and pepstatin (5 $\mu\text{g}/\text{ml}$). Extracts (40 μg) were resolved on 10% Bis-Tris SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes (Invitrogen). CIC was detected with a rabbit antibody against CIC (1:1000) (38) followed by horse-

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radish peroxidase-conjugated anti-rabbit antibody (1:10,000) (Amersham Biosciences). γ -Tubulin was detected by immunoblotting with a mouse antibody against γ -tubulin (1:4000) (Sigma) followed by horseradish peroxidase-conjugated anti-mouse antibody (1:15,000) (Amersham Biosciences). Protein bands were detected with the ECL Advance immunoblot detection kit (Amersham Biosciences).

Islet Isolation and Insulin Secretion—Islets were harvested from adult male Sprague-Dawley rats weighing \sim 250 g, as previously described (13, 18, 28). Islets were incubated overnight in RPMI 1640 medium containing 8 mM glucose and supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 20 units/ml penicillin, 20 μ g/ml streptomycin, and 0.05 μ g/ml amphotericin B (Invitrogen). Insulin secretion was performed with 20 islets for each condition. Islets were washed and incubated for 1 h in KRBH-bovine serum albumin secretion buffer containing 4.38 mM KCl, 1.2 mM $MgSO_4$, 1.5 mM KH_2PO_4 , 129 mM NaCl, 5 mM $NaHCO_3$, 10 mM NaHEPES (Sigma), 3.11 mM $CaCl_2$, 0.25% bovine serum albumin (Sigma), and 2.8 mM glucose. Islets were then incubated for 2 h in secretion buffer containing 2.8 mM glucose or 16.7 mM glucose in the presence or absence of 30 mM KCl. Insulin secretion and insulin content were measured as previously described (35). Cells were treated with 1000 particles/ml of Ad-siCIC or Ad-siControl adenoviruses for 3 days unless otherwise stated.

Islet Perfusion—60 islets were loaded onto a Swinnex 13 column containing a nylon filter (Millipore, Burlington, MA). The chamber was perfused with KRBH-bovine serum albumin buffer with or without the addition of 0.5 or 2 mM BTC and containing various secretagogues at a flow rate of 0.5 ml/min using a Gilson Minipuls 3 pump (France). The temperature was maintained at 37 $^{\circ}C$ using an eight line in-line solution heater (Warner Instruments, Hamden, CT). Islets were perfused at low glucose (2.8 mM) for 45 min prior to each experiment. The solution was gassed with 95% O_2 , 5% CO_2 to achieve a pH of 7.4 and maintained at 37 $^{\circ}C$.

Statistics—Statistical significance was assessed by Student's *t* test or by one-way or two-way analysis of variance for repeated measures followed by multiple Bonferroni comparisons. All data are expressed as means \pm S.E.

RESULTS

The CIC Inhibitor BTC Inhibits GSIS in 832/13 Cells—As a first step in evaluation of the potential role of CIC in regulation of GSIS, we used the substrate analogue 1,2,3-benzenetricarboxylate (BTC; Sigma) to inhibit its activity in robustly glucose-responsive INS-1-derived 832/13 cells. 2.0 mM BTC significantly inhibited both glucose-stimulated (16.7 mM) and glucose + KCl-stimulated (16.7 mM glucose + 30 mM KCl) insulin secretion without affecting basal insulin secretion (Fig. 1A). The effects of BTC on the -fold response to glucose were dose-dependent (Fig. 1B). Treatment of cells with 2 mM BTC resulted in reduction of the -fold response of GSIS from 6.5 ± 1.0 -fold in control (non-drug-treated) cells to 3.9 ± 0.7 -fold. As would be expected of an inhibitor of CIC and mitochondrial citrate export, BTC significantly reduced the incorporation of radiolabeled glucose into the organically extractable lipid fraction (Fig. 1C). Insulin content was slightly decreased in response to 2 mM BTC in 832/13 cells (3052 ± 377

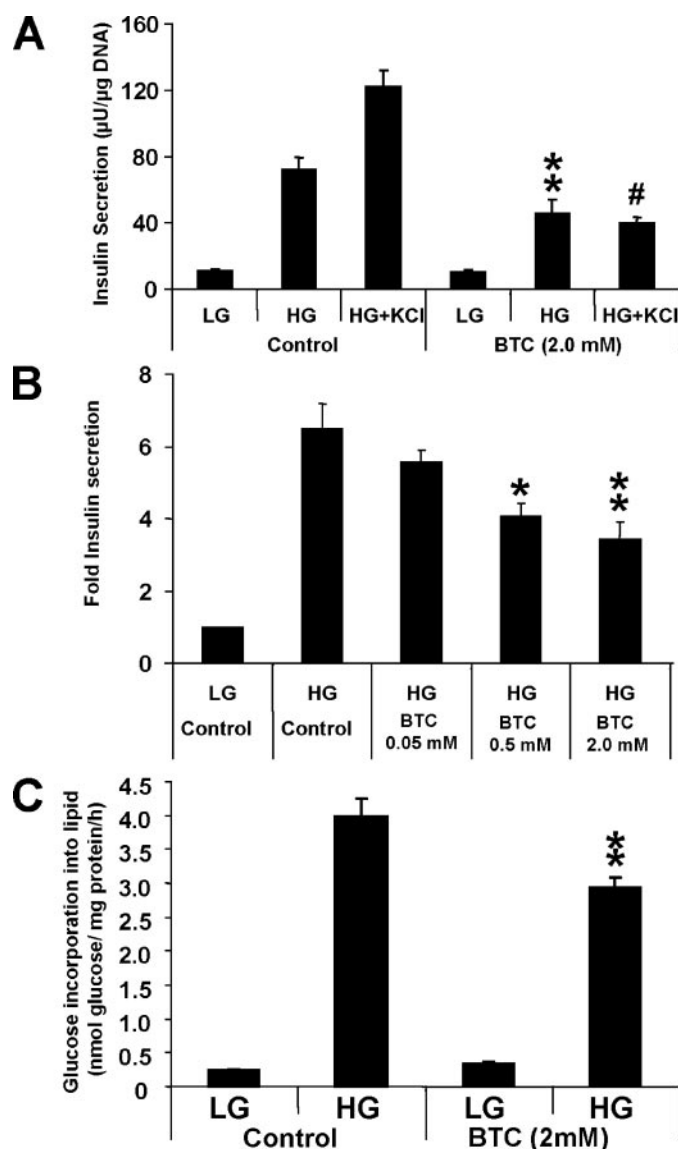


FIGURE 1. The CIC inhibitor BTC inhibits insulin secretion in 832/13 cells. A, insulin secretion in response to low glucose (LG; 2.8 mM), high glucose (HG; 16.7 mM), or high glucose plus KCl (HG + KCl; 16.7 mM glucose + 30 mM KCl). B, BTC dose-dependently inhibits glucose-stimulated insulin secretion. C, the effect of BTC on U- ^{14}C glucose incorporation into lipids. Data represent the mean \pm S.E. for 8–12 independent experiments. For all panels, *, $p < 0.05$; **, $p < 0.01$, HG control versus HG BTC-treated; #, $p < 0.01$ HG + KCl control versus HG + KCl plus BTC.

versus 2571 ± 134 microunits/ μ g of DNA; $p < 0.05$). None of the aforementioned effects of BTC could be ascribed to cytotoxicity, since treatment of 832/13 cells for 3 h with 2 mM BTC had no effect on cell viability, as assessed either by the ToxiLight cytotoxicity assay or the MTS mitochondrial dye method (data not shown).

The CIC Inhibitor BTC Inhibits GSIS in Isolated Rat Islets—We next sought to determine whether BTC inhibits GSIS in primary rat islets. In static incubation experiments, the addition of 2 mM BTC to islets inhibited insulin secretion in response to 16.7 mM glucose or 16.7 mM glucose + 30 mM KCl compared with islets incubated in the absence of the drug (Fig. 2A). To learn more about the effects of BTC on the phases of insulin secretion, we performed islet perfusion studies involv-

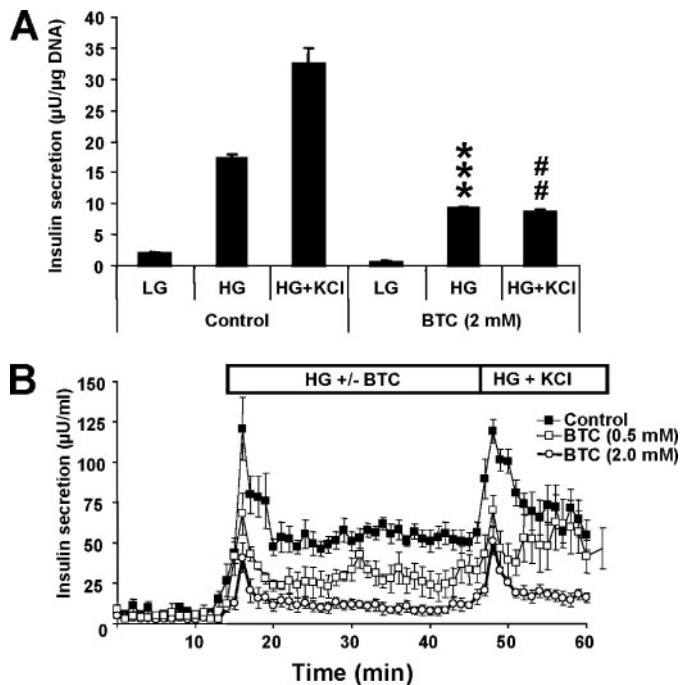


FIGURE 2. The effect of BTC on insulin secretion in isolated rat islets. *A*, insulin secretion measured by static incubation (2 h); *B*, insulin secretion measured by islet perfusion. The islet perfusion studies were performed in the presence or absence of 0.5 or 2 mM BTC, added during stimulation with high glucose (HG; 16.7 mM). Data represent the mean \pm S.E. of 3–6 experiments. ***, $p < 0.001$ HG control versus HG BTC; ##, $p < 0.001$ HG + KCl control versus HG + KCl plus BTC.

ing preperfusion with 2.8 mM glucose for 45 min, followed by 2.8 mM glucose for 15 min, 16.7 mM glucose plus or minus BTC (0.5 or 2 mM) for 30 min, and then 15 min with 16.7 mM glucose + 30 mM KCl in the absence of BTC. These experiments revealed that BTC inhibited both the first and second phases of insulin secretion in response to 16.7 mM glucose (Fig. 2*B*).

Transfection-based siRNA-mediated Suppression of CIC Expression Inhibits GSIS—In order to further investigate the role of CIC in control of GSIS by an alternative, nonpharmacologic approach, 832/13 cells were electroporated with four different siRNA duplexes against CIC or a control, nonspecific siRNA duplex (siControl). The four CIC-specific siRNA duplexes reduced CIC mRNA levels by 50–77% and caused impairment of insulin secretion in response to high glucose to degrees (25–49%) in proportion to their efficacy for knockdown of CIC expression (Fig. 3, *A* and *B*). Treatment of 832/13 cells with CIC siRNA duplexes did not affect insulin content compared with siControl-treated cells (data not shown).

Adenovirus-mediated Delivery of a CIC-specific siRNA Inhibits GSIS—In order to further investigate the effects of CIC knockdown, we constructed a recombinant adenovirus (Ad-siCIC) containing the siRNA sequence corresponding to the most effective duplex siRNA against CIC, based on the data in Fig. 3*A* (duplex CIC 136). Treatment of 832/13 cells with Ad-siCIC caused a virus dose-dependent decrease in CIC mRNA levels compared with cells treated with a control adenovirus (Ad-siControl) (Fig. 4*A*). Treatment of 832/13 cells with Ad-siCIC also resulted in a $53 \pm 8\%$ reduction in CIC protein levels compared with cells treated with Ad-siControl or Ad-βGal (Fig. 4*B*). Based on these studies, two viral doses were selected

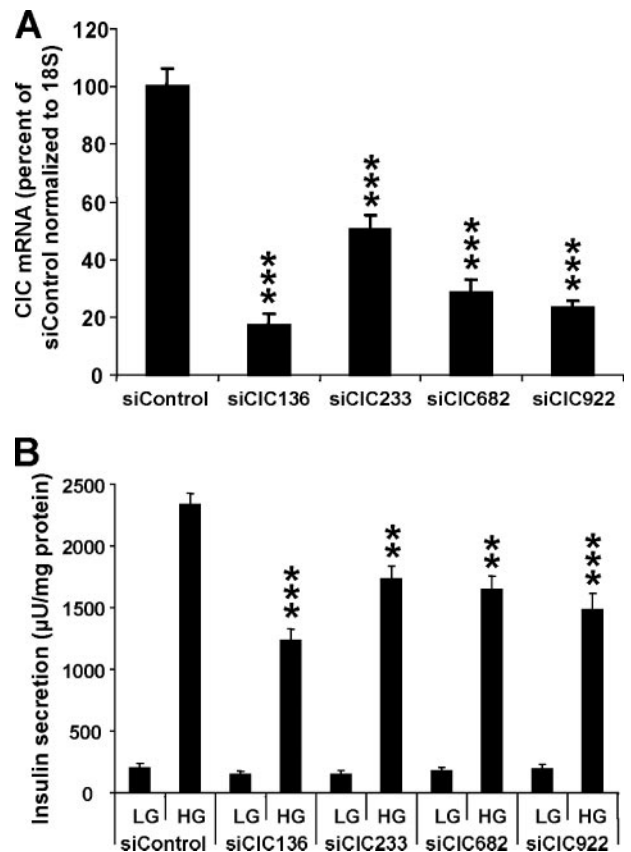


FIGURE 3. Effects of suppression of CIC expression via four different siRNA duplexes in 832/13 cells. Four distinct siRNA duplexes specific for CIC (siCIC136, siCIC233, siCIC682, and siCIC922) or a control siRNA duplex with no known sequence homology (siControl) were introduced into 832/13 cells by electroporation, followed by 48 h of tissue culture before beginning the experiment. *A*, measurement of CIC mRNA levels by real time PCR. *B*, glucose-stimulated insulin secretion. Data represent the mean \pm S.E. for 3–6 independent experiments. **, $p < 0.01$; ***, $p < 0.001$ siControl versus the various siCIC duplexes.

for further experiments, “low” (100 particles/cell), which caused no significant decrease in CIC mRNA levels, and “high” (500 particles/cell), which caused a suppression of $75 \pm 3\%$ relative to Ad-siControl-treated cells. The low dose of Ad-siCIC did not affect glucose- or glucose + KCl-stimulated insulin secretion, whereas the high dose of Ad-siCIC inhibited both glucose-stimulated ($47 \pm 2\%$) and glucose + KCl-stimulated insulin secretion ($45 \pm 2\%$) compared with cells treated with either Ad-siControl or the low dose of Ad-siCIC (Fig. 4*C*). Importantly, insulin secretion in the presence of low glucose + 30 mM KCl was not different in Ad-siCIC compared with Ad-siControl-treated cells (430 ± 14 versus 412 ± 19 microunits/mg of protein; control versus Ad-siCIC), indicating that suppression of CIC expression does not interfere with nutrient-independent stimulation of insulin secretory granule exocytosis.

CIC Suppression Leads to Decreased Cytosolic Citrate Levels—We next sought to confirm our findings of effective siRNA-mediated knockdown of CIC mRNA and protein levels (Figs. 3 and 4) via measurement of the functional activity of CIC in living cells. To this end, we measured total, cytosolic, and mitochondrial citrate levels in 832/13 cells incubated at high glucose and treated with either Ad-siCIC or Ad-siControl. Treatment

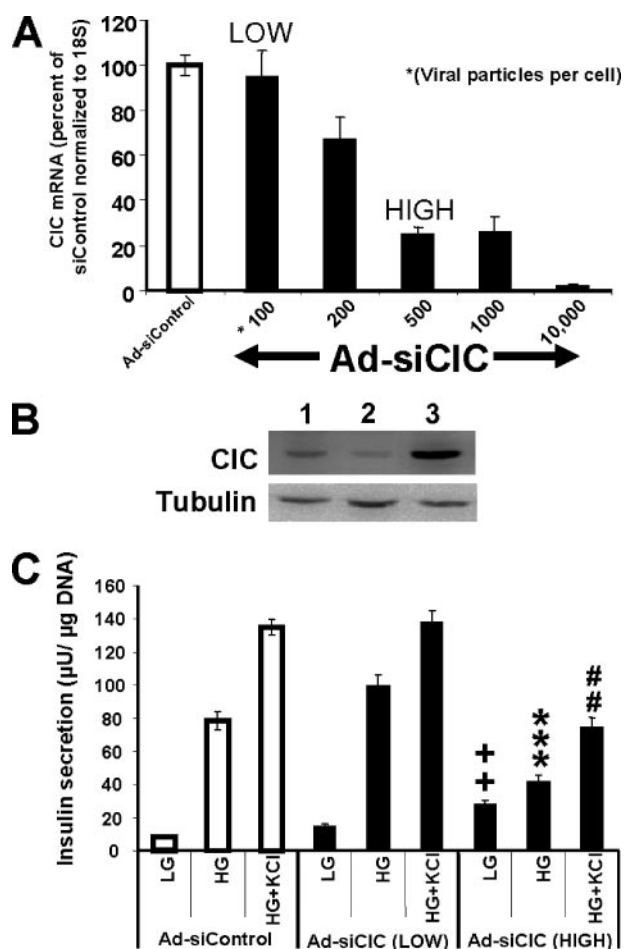


FIGURE 4. Effects of adenovirus-mediated suppression of CIC expression on insulin secretion in 832/13 cells. *A*, dose-dependent effects of Ad-siCIC on CIC mRNA levels, measured by real time PCR. *B*, immunoblot analysis in extracts from cells treated with AdCMV- β -Gal (lane 1), Ad-siCIC (lane 2), or AdCMV-CIC (lane 3). The blot shown is representative of three independent experiments, in which Ad-siCIC treatment caused an average $53 \pm 8\%$ suppression and AdCMV-CIC treatment caused a 10 ± 2 -fold increase in CIC protein levels compared with AdCMV- β -GAL-treated control cells. *C*, dose-dependent effects of Ad-siCIC on insulin secretion. For the secretion studies, the viral titers indicated by the words *low* and *high* in *A* were used, which caused no significant suppression of CIC expression or a $74.7 \pm 3\%$ suppression, respectively. The viral dose of the Ad-siControl was the same as that for the high dose of Ad-siCIC (500 particles/cell). For both panels, data represent the mean \pm S.E. of 8–10 independent experiments. ++, $p < 0.01$ LG Ad-siControl versus LG Ad-siCIC; ***, $p < 0.001$ HG Ad-siControl versus HG Ad-siCIC; ##, $p < 0.01$ HG + KCl Ad-siControl versus HG + KCl Ad-siCIC.

with Ad-siCIC resulted in a $37 \pm 3\%$ decrease in total cellular citrate levels and a $54 \pm 2\%$ decrease in cytosolic citrate concentration, with no change in citrate content of intact mitochondria (Fig. 5, *A* and *B*). Cytosolic and mitochondrial citrate levels were discriminated by selective permeabilization of the plasma membrane with a low concentration of saponin ($80 \mu\text{g/ml}$) (36, 37). The validity of the assay is supported by our finding of recovery of 90% of total citrate lyase activity and $<1\%$ of total citrate synthase in the cytosolic fraction (data not shown). These data are consistent with a significant decrease in the expression and functional activity of CIC, the major conduit for citrate export from mitochondria.

Metabolic Effects of CIC Suppression—In an effort to gain insight into potential metabolic mechanisms linking suppression of CIC expression to impairment of GSIS, we meas-

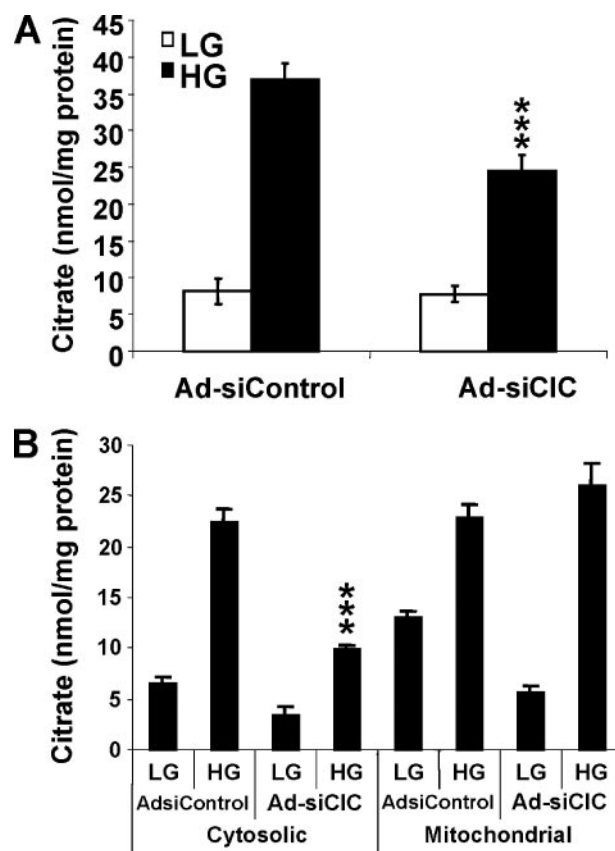


FIGURE 5. Treatment of 832/13 cells with Ad-siCIC reduces total and cytosolic citrate levels. *A*, total cellular citrate levels; *B*, citrate levels in cytosolic and mitochondrial cell fractions, prepared as described under "Experimental Procedures." Data represent the mean \pm S.E. of 3–5 independent experiments. ***, $p < 0.001$ HG Ad-siControl versus HG Ad-siCIC.

ured a wide array of metabolic variables. Treatment of 832/13 cells with the high dose of Ad-siCIC adenovirus did not affect the glycolytic rate (Fig. 6*A*) or glucose oxidation (Fig. 6*B*) relative to Ad-siControl treatment but did reduce the incorporation of radiolabeled glucose into fatty acids by $34 \pm 5\%$ (Fig. 6*C*). Neither ATP or ADP levels were significantly altered by Ad-siCIC treatment, and glucose caused identical increases in ATP/ADP ratio in Ad-siCIC relative to Ad-siControl-treated cells (data not shown). Consistent with the latter finding, glucose-stimulated hyperpolarization of the mitochondrial membrane potential was not changed in Ad-siCIC compared with Ad-siControl-treated cells (Ad-siControl-treated cells, $78 \pm 10\%$; Ad-siCIC-treated cells, $84 \pm 6\%$ fluorescence at 20 mM glucose relative to fluorescence at 2.8 mM glucose set to 100% for both groups). Finally, recent studies have suggested that pyruvate/citrate and pyruvate/isocitrate cycling may be linked to GSIS via production of NADPH in the ICDC reaction (18). Consistent with this idea, Ad-siCIC treatment resulted in lowering of NADPH and NADP^+ levels at both low and high glucose levels but with a larger effect on NADPH, such that the NADPH/ NADP^+ ratio at stimulatory glucose and the increment in NADPH/ NADP^+ as glucose was raised from low to high levels was significantly reduced in Ad-siCIC-treated compared with Ad-siControl-treated cells (Fig. 7, *A* and *B*).

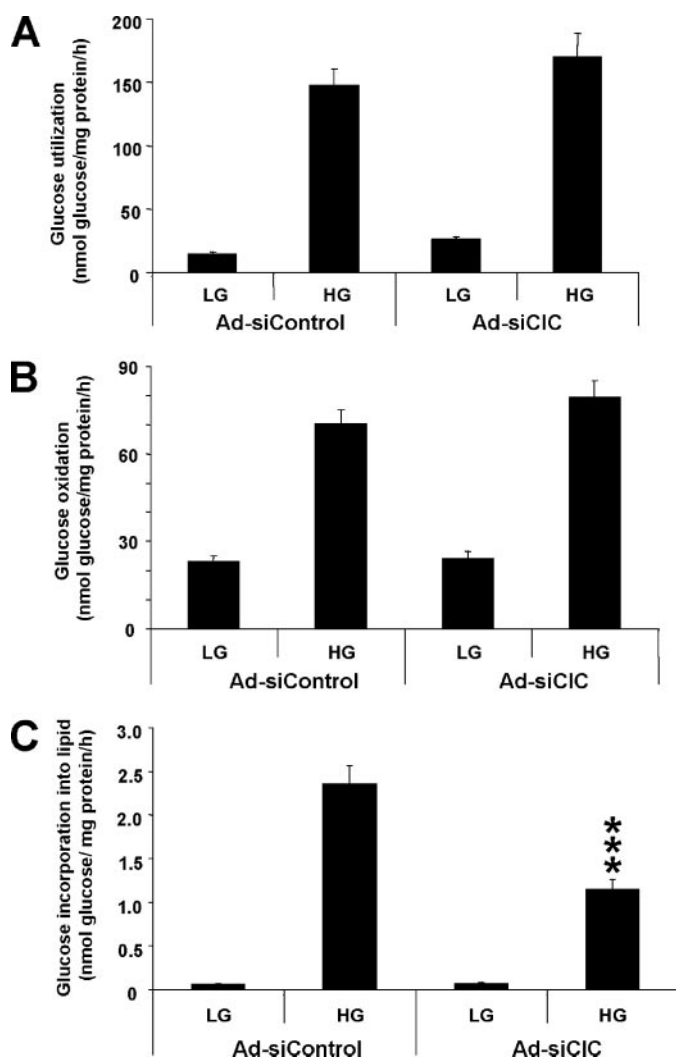


FIGURE 6. Metabolic effects of Ad-siCIC treatment in 832/13 cells. A, glycolytic rate; B, glucose oxidation; C, $U\text{-}^{14}\text{C}$ glucose incorporation into fatty acids. Data represent the mean \pm S.E. of 3–4 independent assays. ***, $p < 0.001$, HG Ad-siControl versus HG Ad-siCIC.

Overexpression of CIC Enhances GSIS and Raises Cytosolic Citrate Levels—Treatment of 832/13 cells with a recombinant adenovirus containing the rat CIC cDNA (AdCMV-CIC) resulted in a 10 ± 2 -fold increase in CIC protein levels as compared with cells treated with AdCMV- β GAL (Fig. 4B). AdCMV-CIC treatment had no significant effect on insulin secretion at low glucose but caused a 60% increase in secretion at stimulatory glucose relative to AdCMV- β GAL-treated cells (Fig. 8A). In addition, AdCMV-CIC treatment increased cytosolic citrate levels in 832/13 cells by 40% compared with AdCMV- β Gal-treated cells (Fig. 8B).

Modulation of CIC Expression in Rat Islets Regulates GSIS—Finally, we tested the effect of manipulation of CIC expression on GSIS in primary rat islets, facilitated by use of a recombinant adenovirus that allows us to deliver the CIC siRNA construct to such cells with high efficiency (28). Treatment of rat islets with Ad-siCIC reduced CIC mRNA levels by $55 \pm 9\%$ ($p < 0.001$) compared with Ad-siControl-treated islets. This amount of CIC knockdown resulted in a $44 \pm 3\%$ inhibition of glucose-stimulated and a $32 \pm 4\%$ inhibition of glucose + KCl-stimu-

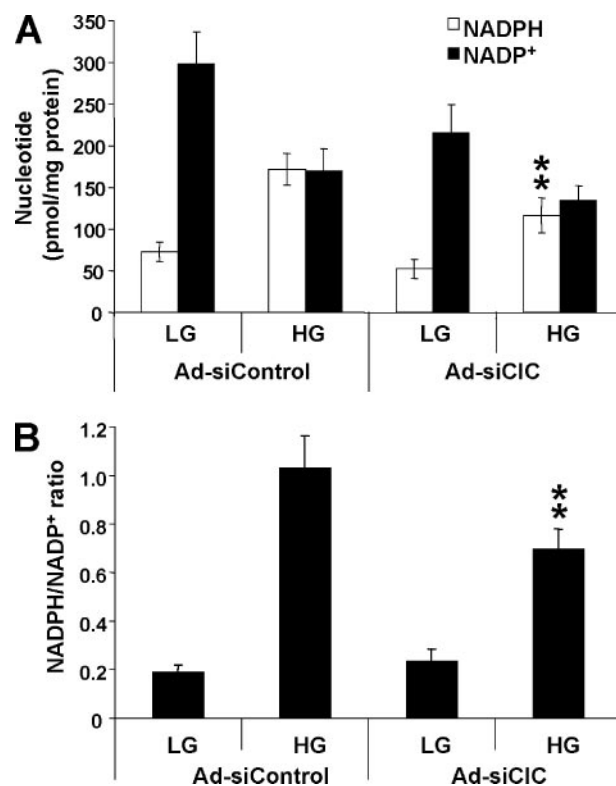


FIGURE 7. The effects of Ad-siCIC on glucose-stimulated alterations in NADPH, NADP⁺, and NADPH/NADP⁺ ratio in 832/13 cells. A, NADPH and NADP⁺ levels in response to LG and HG. **, $p < 0.01$, NADPH HG Ad-siControl versus NADPH HG Ad-siCIC. B, NADPH/NADP⁺ ratio. **, $p < 0.01$, HG Ad-siControl versus HG Ad-siCIC. Data represent the mean \pm S.E. of five independent assays.

lated insulin secretion in isolated rat islets (Fig. 9). Ad-siCIC treatment did not affect islet insulin content relative to Ad-siControl-treated islets (data not shown).

DISCUSSION

The studies summarized herein demonstrate that the mitochondrial tricarboxylate or citrate/isocitrate carrier (CIC) plays an important role in GSIS. Inhibition of CIC activity by the specific substrate analogue BTC resulted in inhibition of GSIS in 832/13 cells. BTC also inhibited first- and second-phase insulin secretion in isolated rat islets. The findings obtained with a pharmacologic tool for suppression of CIC were confirmed by molecular approaches. Thus, delivery of siRNA constructs specific for CIC either by duplex transfection or in the context of a recombinant adenovirus (Ad-siCIC) caused a clear decrease in CIC mRNA and protein levels and impaired GSIS in 832/13 cells. Knockdown of CIC was without effect on glucose utilization, glucose oxidation, or ATP/ADP ratio but did cause significant lowering of glucose-stimulated citrate accumulation in the cytosol and glucose incorporation into lipids in these cells. Conversely, CIC overexpression resulted in increased accumulation of cytosolic citrate and enhanced GSIS in 832/13 cells. Finally, adenovirus-mediated suppression of CIC expression in primary rat islets impaired GSIS. These findings support the concept that the ability of glucose to stimulate an increase in cytosolic citrate or isocitrate levels plays an important role in control of GSIS, independent of changes in ATP/ADP ratio. It should also be noted that the lack of effect of CIC

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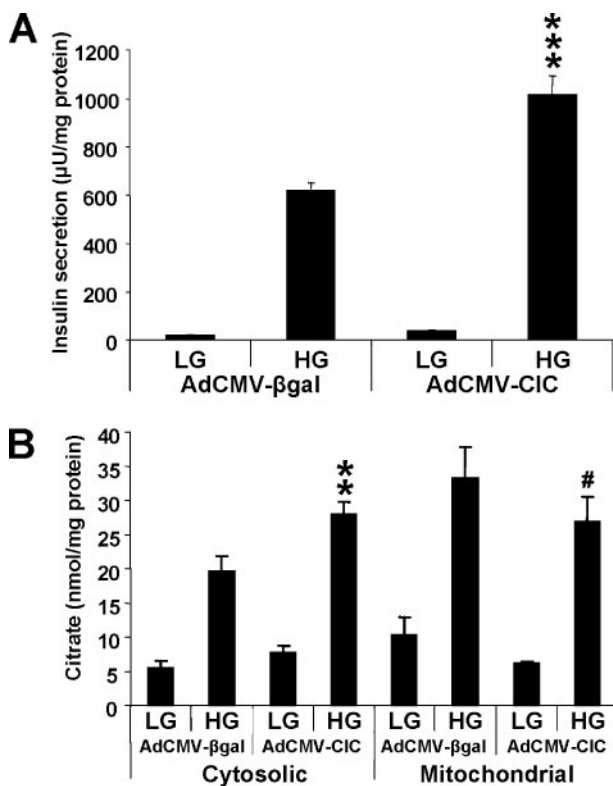


FIGURE 8. CIC overexpression virus increases GSIS and cytosolic citrate levels in 832/13 cells. *A*, glucose-stimulated insulin secretion. *B*, citrate levels in cytosolic and mitochondrial cell fractions, prepared as described under "Experimental Procedures." Data represent the mean \pm S.E. of 3–5 independent experiments. **, $p < 0.01$; ***, $p < 0.001$ HG AdCMV-βGal versus HG AdCMV-CIC; #, $p < 0.05$ HG AdCMV-βGal versus HG AdCMV-CIC (mitochondrial citrate).

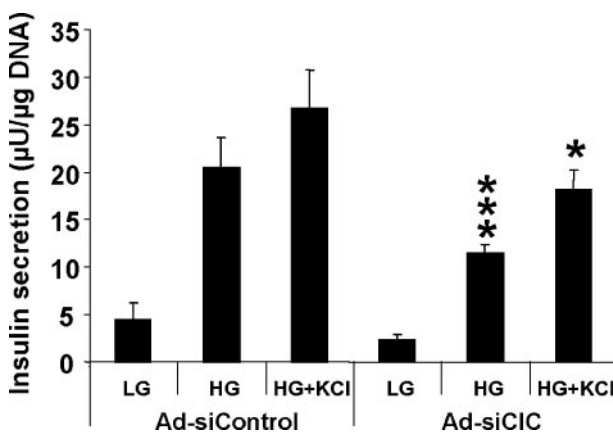


FIGURE 9. Ad-siCIC treatment of primary rat islets. Freshly harvested rat islets were treated with Ad-siControl or Ad-siCIC for 2 h, and GSIS was measured 72 h after viral treatment. Data represent the mean \pm S.E. for four independent experiments. *, $p < 0.05$; ***, $p < 0.001$ HG Ad-siControl versus HG Ad-siCIC.

knockdown on glycolytic flux or glucose oxidation argues against a nonspecific or global effect of CIC knockdown on β -cell glucose metabolism, as does our finding of no change in cell viability using the MTS assay, which provides an index of mitochondrial function.

To date, alteration in ATP/ADP ratio is the only universally accepted pathway that links glucose metabolism to insulin secretion (12). However, it is clear from numerous studies that

glucose retains an ability to stimulate insulin secretion even when ATP-sensitive K^+ channels are rendered unresponsive, strongly implying that metabolic signals other than ATP/ADP ratio are important for GSIS (4, 11, 12). Several by-products of mitochondrial metabolism of glucose have been invoked as candidates for mediating this ancillary signaling pathway, including glutamate, malonyl-CoA, LC-CoA, and NADPH (7–10, 14, 24, 39). The current study provides clear evidence that coupling factors derived from the metabolism of citrate or isocitrate deserve particular scrutiny.

Technically, citrate or isocitrate can be converted to α -keto-glutarate, which can in turn be converted to glutamate by glutamate dehydrogenase or transaminases. However, studies in several laboratories have failed to identify increases in glutamate levels during glucose stimulation of β -cells (8, 9, 40). Moreover, glutamate probably serves as an insulin secretagogue via glutaminolysis or oxidation of glutamate in the TCA cycle (41). Producing glutamate from citrate or isocitrate would thus seem to be an inefficient and unlikely mechanism for explaining our results.

The malonyl-CoA/LC-CoA model of GSIS holds that during glucose stimulation, pyruvate carboxylase-mediated anaplerosis raises cytosolic citrate levels, which leads in turn to an increase in malonyl-CoA levels (16, 17). Malonyl-CoA is a potent inhibitor of carnitine palmitoyltransferase I (42), and this action could divert LC-CoA away from oxidation in the mitochondria toward accumulation in the cytosol (16, 17). Consistent with this model, treatment of β -cells with glucose causes a rapid rise in malonyl-CoA levels that precedes insulin secretion (16). Glucose stimulation also suppresses fatty acid oxidation, and the addition of LC-CoA stimulates insulin granule exocytosis in permeabilized β -cells (43). However, LC-CoA also stimulates K_{ATP} channel activity in patch-clamped β -cells (44, 45), an effect seemingly at odds with a role of LC-CoA as a glucose-derived stimulus/secretion coupling factor. Furthermore, prevention of the glucose-induced rise in malonyl-CoA levels by overexpression of malonyl-CoA decarboxylase has no impact on GSIS (33, 46), a finding recently confirmed by the laboratory that developed the malonyl-CoA/LC-CoA hypothesis (47). Similarly, treatment of β -cells with Triacsin C, an inhibitor of LC-CoA synthetase, does not impair glucose responsiveness (33, 46). In a modification of the original hypothesis, it has recently been suggested that malonyl-CoA/LC-CoA might be important for the potentiating effect of fatty acids on GSIS, since experiments with triacsin C and malonyl-CoA decarboxylase overexpression diminished this action of fatty acids in β -cell lines and rat islets (47), although different results were obtained by another laboratory with malonyl-CoA decarboxylase (46). Overall, there is now a consistent lack of evidence for a direct role of malonyl-CoA in regulation of GSIS, whereas its potential role in lipid-mediated potentiation of GSIS remains an open question. Since lipids were not included in our analysis of insulin secretion in the present study, it is unlikely that the effect of CIC blockade to suppress GSIS is due to lowering of malonyl-CoA levels.

Among the current candidates for the ancillary mitochondrially derived signal for insulin secretion, a role for NADPH is supported by several lines of evidence. First, pyruvate cycling

activity is strongly correlated with the capacity for GSIS in a panel of INS-1-derived cell lines, and NADPH is an expected by-product of all of the proposed pyruvate cycling pathways (8, 9, 13, 18–21). Second, the NADPH/NADP⁺ ratio increases in direct proportion to media glucose concentration and GSIS in rodent islets and several β -cell lines, whereas this relationship does not exist for NADH/NAD⁺ ratio and GSIS (15, 18). Third, the addition of NADPH to patch-clamped β -cells stimulated exocytosis as measured by increases in cell capacitance, whereas NADH had no effect. These studies also suggest that NADPH/NADP⁺ ratio may be the relevant signal, since the addition of NADP⁺ reversed the stimulatory effect obtained with NADPH alone (15). Finally, suppression of ICDC expression by adenovirus-mediated delivery of a siRNA construct caused a coordinate reduction in pyruvate cycling activity and NADPH/NADP⁺ ratio and simultaneously caused strong impairment of GSIS in β -cell lines and rat islets (18). The findings of the current study fully support the NADPH/NADP⁺ ratio model, since we found that siRNA-mediated knockdown of CIC caused significant decreases in NADPH levels as well as a reduction in the increment in NADPH/NADP⁺ ratio as glucose was raised from low to high levels.

If NADPH is an important signaling molecule for insulin secretion, the targets by which it mediates its effects remain to be identified (8). One interesting candidate appears to be voltage-gated K⁺ channels, which were recently shown to be regulated by changes in NADPH/NADP⁺ levels (48). However, it also remains possible that non-NADPH-related byproducts of citrate and/or isocitrate metabolism in the cytosol are the critical pyruvate cycling-related factors that regulate insulin secretion. In fact, a signaling role for cytosolic α -ketoglutarate or an intermediate produced from its further metabolism has been suggested by a recent study (49). In any case, the finding that suppression of CIC (current study) or ICDC (18) expression causes clear impairment of GSIS focuses immediate effort on gaining more insight into by-products of citrate and isocitrate metabolism in the β -cell, including NADPH.

In conclusion, mitochondrial CIC not only occupies a critical position in intermediary metabolism but also plays an important role in GSIS. Our studies further highlight the importance of anaplerotic metabolism of glucose and pyruvate cycling pathways as key mediators of GSIS and suggest that enzymes and proteins that regulate pyruvate cycling should be evaluated for their potential role in β -cell failure of diabetes or as targets for enhancing β -cell function.

Acknowledgments—We thank Dr. Danhong Lu for assistance with islet isolation and Helena Winfield, Lisa Poppe, and Paul Anderson for expert technical support.

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**The Mitochondrial Citrate/Isocitrate Carrier Plays a Regulatory Role in
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J. Biol. Chem. 2006, 281:35624-35632.

doi: 10.1074/jbc.M602606200 originally published online September 25, 2006

Access the most updated version of this article at doi: [10.1074/jbc.M602606200](https://doi.org/10.1074/jbc.M602606200)

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