

Normal Flux through ATP-Citrate Lyase or Fatty Acid Synthase Is Not Required for Glucose-stimulated Insulin Secretion*

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It has been proposed that *de novo* synthesis of long-chain acyl-CoA (LC-CoA) is a signal for glucose-stimulated insulin secretion (GSIS). Key enzymes involved in synthesis of fatty acids from glucose include ATP-citrate lyase (CL) and fatty acid synthase (FAS). An inhibitor of CL, hydroxycitrate (HC), has been reported to inhibit insulin secretion in some laboratories but not in others. Here we show that high concentrations of NaCl created during preparation of HC by standard methods explain the inhibition of GSIS, and that removal of the excess NaCl prevents the effect. To further investigate the role of CL, two small interfering RNA adenoviruses (Ad-siCL2 and Ad-siCL3) were generated. Ad-siCL3 reduced CL mRNA levels by $92 \pm 6\%$ and CL protein levels by $75 \pm 4\%$ but did not affect GSIS in 832/13 cells compared with cells treated with a control adenovirus (Ad-siControl). Similar results were obtained with Ad-siCL2. Ad-siCL3-treated cells also exhibited a $52 \pm 7\%$ reduction in cytosolic oxaloacetate, an $83 \pm 4\%$ reduction in malonyl-CoA levels, and inhibition of [U - ^{14}C]glucose incorporation into lipid by $43 \pm 4\%$, all expected metabolic outcomes of CL suppression. Similarly, treatment of 832/13 cells with a recombinant adenovirus specific to FAS (Ad-siFAS) reduced FAS mRNA levels by $81 \pm 2\%$ in 832/13 cells, resulting in a $59 \pm 4\%$ decrease in [U - ^{14}C]glucose incorporation into lipid, without affecting GSIS. Finally, treatment of primary rat islets with Ad-siCL3 or Ad-siFAS reduced CL and FAS mRNA levels by $65 \pm 4\%$ and $52 \pm 3\%$, respectively, but had no effect on GSIS relative to Ad-siControl-treated islets. These findings demonstrate that a normal rate of flux of glucose carbons through CL and FAS is not required for GSIS in insulinoma cell lines or rat islets.

The mechanisms that control glucose-stimulated insulin secretion (GSIS)² from pancreatic islet β -cells are incompletely

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² The abbreviations used are: GSIS, glucose-stimulated insulin secretion; CL, ATP-citrate lyase; LC-CoA, long-chain acyl-CoA; PC, pyruvate carboxylase; ICDC, cytosolic isocitrate dehydrogenase; FAS, fatty acid synthase; siRNA,

understood. One glucose-derived signal appears to be a rise in the ATP:ADP ratio, which stimulates closure of ATP-sensitive K^+ (K_{ATP}) channels, resulting in plasma membrane depolarization, activation of voltage-gated Ca^{2+} channels, and Ca^{2+} -mediated stimulation of insulin granule exocytosis (1–4). This so-called " K_{ATP} channel-dependent" mechanism appears to be particularly important in triggering exocytosis of a small number of granules from a plasma membrane-docked "readily releasable pool" responsible for the first, acute phase of insulin release (5). In contrast, in the second and sustained phase of insulin secretion, ATP and Ca^{2+} may play only limited or permissive roles, allowing other glucose-derived second messengers to come to the forefront (5). Important support for the K_{ATP} channel-independent pathways of β -cell glucose signaling came from studies showing that glucose causes a significant increase in insulin secretion even when K_{ATP} channels are held open by application of diazoxide and high K^+ , or in animals in which the SUR1 K^+ channel was knocked out (5, 6). These and more recent studies suggest that mitochondrial metabolism of glucose generates signals other than changes in the ATP:ADP ratio that are important for control of insulin secretion (3, 4, 7–16).

Understanding the nature of all of the glucose-derived signals for insulin secretion (both K_{ATP} channel-dependent and -independent) will be required to understand the functional failure of the β -cell in diabetes and for the development of new drugs for correcting this problem. Recent studies in this area have focused on understanding the metabolic fates of pyruvate in the mitochondria. Islet β -cells express both pyruvate carboxylase (PC) and pyruvate dehydrogenase in abundance, such that in the fed state, pyruvate flows into mitochondrial metabolic pathways in roughly equal proportions through the anaplerotic (PC) and oxidative (pyruvate dehydrogenase) entry points (7, 17–19). Furthermore, islet β -cells contain enzymes that allow "cycling" of pyruvate via its PC-catalyzed conversion to oxaloacetate, metabolism of oxaloacetate to malate, citrate, or isocitrate in the TCA cycle, and subsequent recycling of these metabolites to pyruvate via several possible combinations of cytosolic and mitochondrial pathways (14, 15, 20). It is now

small interfering RNA; α -KG, α -ketoglutarate; FAS, fatty acid synthase; HC, hydroxycitrate; KRB, Krebs-Ringer bicarbonate buffer; HG, high glucose (16.7 mM); LG, low glucose (2.8 mM).

clear that pyruvate cycling is important in the regulation of GSIS (7, 14–16, 21, 22). Thus, GSIS is correlated with PC-catalyzed pyruvate cycling, but not with pyruvate dehydrogenase-catalyzed glucose oxidation in variously glucose responsive INS-1-derived cell lines, and inhibition of pyruvate cycling activity with PC inhibitors impairs GSIS (7, 23).

More recent studies have focused on identification of the specific pyruvate cycling pathways that generate signals for insulin secretion. One important step is the export of citrate and/or isocitrate from the mitochondria via the citrate/isocitrate carrier. Pharmacologic or siRNA-mediated suppression of citrate/isocitrate carrier causes substantial impairment of GSIS in both insulinoma cell lines and primary rat islets (16). Once exported to the cytosol, citrate and isocitrate can recycle to pyruvate either via ATP-citrate lyase (CL)-mediated cleavage to oxaloacetate and acetyl-CoA, or via cytosolic NADP⁺-dependent isocitrate dehydrogenase (ICDc)-mediated conversion to α -ketoglutarate (α -KG) and recycling to pyruvate via mitochondrial enzymes. Support for an important role of the latter pathway comes from our recent demonstration that siRNA-mediated suppression of ICDc impairs GSIS in insulinoma cells and primary rat islets, suggesting that a metabolic byproduct of pyruvate/isocitrate cycling plays an important role in control of GSIS (15).

Our findings to date do not preclude an independent contribution by a pyruvate/citrate cycling pathway that is initiated by citrate cleavage catalyzed by CL. Indeed, others have proposed a “malonyl-CoA/long-chain acyl-CoA” model of GSIS that holds that PC-mediated anaplerosis is linked to control of insulin secretion via pyruvate/citrate cycling and increases in the levels of byproducts of this pathway, malonyl-CoA, and long-chain acyl-CoAs (LC-CoA) (8, 9). Consistent with this model, treatment of β -cells with glucose causes a rapid rise in malonyl-CoA levels that precedes insulin secretion (8). Glucose stimulation also suppresses fatty acid oxidation, and addition of LC-CoA stimulates insulin granule exocytosis in permeabilized β -cells (24). However, LC-CoA also stimulates K_{ATP} channel activity in patch-clamped β -cells (25, 26), 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 (27–29).

Although malonyl-CoA may not be the direct signal for GSIS, other products of a pyruvate/citrate cycling or lipogenesis pathway initiated by CL cleavage of citrate could play an important role. In the current study, we have investigated the effects of pharmacologic and siRNA-mediated inhibition of CL and fatty acid synthase (FAS) activity in insulinoma cells and primary rat islets. We find that suppression of CL activity in β -cells leads to anticipated metabolic effects, including a fall in cytosolic oxaloacetate and malonyl-CoA levels, and both CL and FAS suppression are accompanied by a decrease in [U-¹⁴C]glucose incorporation into lipids. However, CL or FAS suppression have no impact on GSIS. Moreover, we show that previous reports of impairment of GSIS by the CL inhibitor hydroxycitrate were likely influenced by an artifact of very high NaCl levels generated during preparation of hydroxycitrate by stand-

ard methods. Elimination of pyruvate/citrate cycling and lipogenesis as important pathways for control of GSIS focuses future work on other pyruvate cycling pathways, most notably the pyruvate/isocitrate and pyruvate/malate cycles.

EXPERIMENTAL PROCEDURES

Cell Lines—The INS-1-derived cell line 832/13, which exhibits robust GSIS, was used in these studies (30). Cells were cultured in RPMI 1640 medium containing 11.1 mM glucose and supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol at 37 °C in a humidified atmosphere containing 5% CO₂.

Preparation of Hydroxycitrate—Hydroxycitrate (HC) was solubilized as recommended by the manufacturer (Sigma). Initially a 5 mM stock solution of HC was prepared by solubilization in 10 N HCl and normalization of pH to \sim 7.0 with 10 N NaOH. This solution was then diluted to the appropriate concentration (0.3 or 1 mM of HC) in Krebs-Ringer bicarbonate buffer (KRB) and the pH adjusted to 7.4 with 0.1 N NaOH. In the process of making a 1 mM solution of HC in KRB the NaCl concentration increased by \sim 150–172 mM due to the use of strong HCl and NaOH for solubilizing and neutralizing the HC solution. In some experiments, we adjusted for this additional NaCl by subtracting NaCl from the KRB buffer.

siRNA Duplexes and Viral Construction—Two siRNA duplexes against ATP-CL (accession number NM016987) were used for adenovirus construction. Relative to the start codon, the 5' end of the siRNA target sequence corresponded to the following nucleotides in CL: CL2, nucleotide 620 (GCTTCATCTCCGGCCTATT); CL3, nucleotide 1027 (AGTCATAGGTCTGTTGTTTC). For FAS the siRNA target sequence corresponded to nucleotide 351 (GCUAUUGUGGACGGAGGUATT). Recombinant adenoviruses were generated containing the siRNA sequences for CL2, CL3, and FAS as previously described (31–33). A recombinant adenovirus containing an siRNA sequence with no known gene homology (Ad-siControl) was constructed as previously described (15, 16, 32) and used as a control.

Real-time PCR Analysis of CL and FAS mRNA Expression—RNA isolation, reverse transcription, and real-time PCR analysis was performed on extracts of 832/13 cells and isolated islets as previously described (15, 16). CL, FAS, and 18S real-time probes were purchased from Applied Biosystems.

CL and FAS Immunoblots—CL and FAS protein were detected using standard immunoblotting techniques. Cellular proteins were extracted with cell lysis buffer (Cell Signaling) containing phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (10 μ g/ml), aprotinin (10 μ g/ml), and pepstatin (5 μ g/ml). Extracts (40 μ g) were resolved on 3–8% Tris acetate/SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes (Invitrogen). CL was detected with an antibody against CL (Cell Signaling), FAS with an antibody specific to FAS (Santa Cruz Biotechnology, CA), and tubulin with an antibody specific to γ -tubulin (Sigma).

CL Enzymatic Activity—CL enzyme activity was measured by a malate dehydrogenase-coupled method (34). Cell lysates were added to a reaction mixture containing 100 mM Tris-HCl (pH 8.7), 20 mM potassium citrate, 10 mM MgCl₂, 10 mM dithiothre-

ATP Citrate Lyase, FAS, and Glucose-stimulated Insulin Secretion

itol, 0.5 units/ml malate dehydrogenase, 0.33 mM CoASH, 0.14 mM NADH, and 5 mM ATP (all from Sigma). Change in absorbance at 340 nm was monitored every 15 s over 20 min in a SpectraMax 190 spectrophotometer. Change in absorbance in the absence of exogenous ATP was subtracted from change in the presence of ATP and was normalized to protein concentration to determine the specific CL activity.

Glucose Incorporation into Lipids—Incorporation of [$U\text{-}^{14}\text{C}$]glucose into cellular lipids was measured as previously described (15, 16, 27).

Cytosolic Oxaloacetate and Total Malonyl-CoA Measurements—A saponin-based method was employed to prepare cytosolic fractions of 832/13 cells as previously described (16). Briefly, cells (5×10^6) were washed twice in phosphate-buffered saline, 0.02% EDTA and then permeabilized in 100 mM KCl, 22 mM NaCl, 5 mM K_2HCO_3 , 10 mM K-HEPES, 1 mM MgCl_2 , and 80 $\mu\text{g/ml}$ saponin (pH 7.4) for 5 min. Cells were mixed and then centrifuged for 3 min at $3000 \times g$. The supernatant (cytosolic fraction) was used to measure oxaloacetate as described previously (35). Protein was precipitated from the cytosolic fraction by addition of 0.25 M perchloric acid at 4°C for 20 min, followed by neutralization with 0.94 M KOH. The perchloric acid precipitate was removed by centrifugation. Supernatant or oxaloacetate standard in perchloric acid (0.2–2.0 pmol) was added to 200 μl of reaction buffer (75 mM K_2HPO_4 , pH 7.4, 80 nM [acetyl- ^3H]acetyl-CoA, 50 $\mu\text{g/ml}$ citrate synthase) at room temperature for 60 min. The reaction was stopped by addition of 600 μl of charcoal mixture (8 g of charcoal, 38 g of citric acid monohydrate, 120 ml of 95% ethanol) followed by centrifugation at $12,000 \times g$ and liquid scintillation counting of the supernatant.

Total cellular malonyl-CoA levels were assayed as previously described (27, 28, 36). Briefly, 832/13 cells (5×10^6) were washed 2 times in phosphate-buffered saline, 0.02% EDTA and extracted with 6% perchloric acid followed by neutralization with 1.67 M KHCO_3 . Assays were performed in 0.2 ml of 0.2 M potassium phosphate buffer (pH 7.2) containing 0.2 mM EDTA, 2.5 mM dithiothreitol, 1 mg/ml fatty acid-free albumin, 0.25 mM NADPH, 0.68 μM [^3H]acetyl-CoA (0.5 $\mu\text{Ci/nmol}$), and either 150 μl of HClO_4 -soluble 832/13 cell extract or 0, 1, 100, 400, or 1000 pmol of malonyl-CoA standard. The reaction was initiated by adding fatty acid synthase, followed by incubation for 2 h at 37°C . After the 2-h incubation, the lipid soluble fraction was extracted and counted by scintillation.

Pyruvate Cycling Measured by ^{13}C NMR—832/13 cells were transfected with siCL3 or a control siRNA duplex as described (15, 16), cultured for 72 h, and then incubated with [$U\text{-}^{13}\text{C}$]glucose for 4 h. Cell extracts were used for NMR-based mass isotopomer analysis and calculation of pyruvate cycling activity as previously described (7, 14, 15, 21).

Islet Isolation and Insulin Secretion—Islets were harvested from male Wistar rats, treated with recombinant adenoviruses, and subjected to insulin secretion assays (static incubation and perfusion) as previously described (16).

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

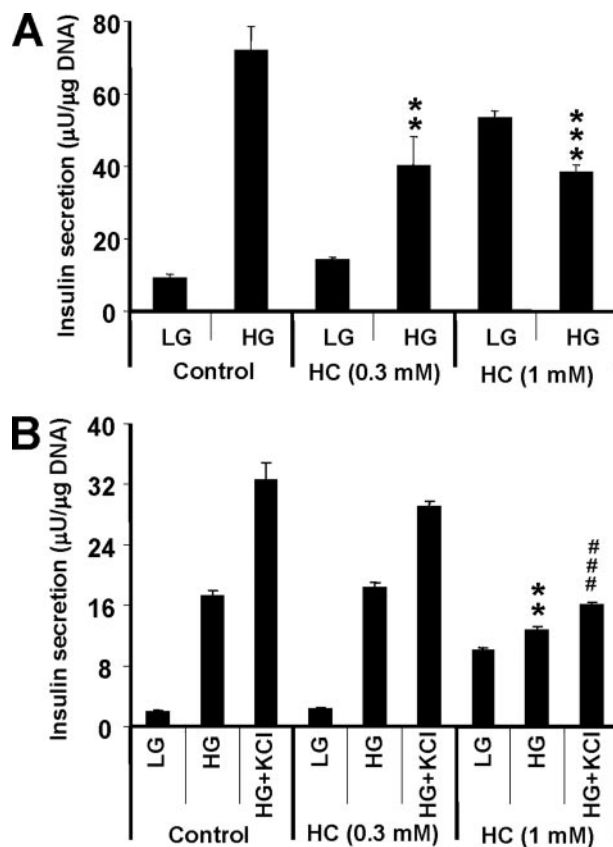


FIGURE 1. Inhibitory effect of HC on GSIS with no correction for NaCl. HC was prepared by standard methods, resulting in a large increase in NaCl concentration (see text for explanation). All data in this figure use the standard HC preparation without correction for increased salt concentration. *A*, the effects of HC on GSIS in 832/13 cells ($n = 5$). *B*, the effects of HC on GSIS in isolated rat islets ($n = 4$). **, $p < 0.01$; ***, $p < 0.001$ HG control versus HG plus HC; ###, $p < 0.01$ HG + KCl control versus HG + KCl plus HC. Low glucose (LG; 2.8 mM), high glucose (HG; 16.7 mM) or high glucose plus KCl (HG+KCl; 16.7 mM glucose and 30 mM KCl) are shown.

RESULTS

The Effects of HC on GSIS in 832/13 Cells and Isolated Rat Islets—Previous studies have reported that GSIS is impaired by HC, an inhibitor of CL (37). The best solvent for HC is a strong acid such as HCl, but use of this reagent then requires neutralization with a strong base (NaOH). In a first set of experiments, 0.3 or 1 mM HC solutions were prepared with no compensation for the extra NaCl that accumulates. In 832/13 cells, 0.3 mM HC inhibited insulin secretion at 16.7 mM glucose by $44 \pm 7\%$ without affecting basal insulin secretion, whereas 1 mM HC caused a $275 \pm 5\%$ increase in basal insulin secretion and a $46 \pm 5\%$ impairment in insulin secretion at 16.7 mM glucose, effectively eliminating GSIS (Fig. 1A). In isolated rat islets 1 mM HC increased basal insulin secretion by $537 \pm 10\%$ and inhibited insulin secretion at 16.7 mM glucose by $33 \pm 5\%$ (Fig. 1B).

The increase in NaCl concentration caused by solubilization of HC in strong HCl and neutralization with NaOH was calculated to be 150–172 mM. When this amount of NaCl was removed from the KRB, 0.25 and 0.5 mM HC inhibited glucose incorporation into lipids by 18.4 ± 6.2 and $47.6 \pm 4.2\%$, respectively (Fig. 2A), but had no effect on GSIS in 832/13 cells (Fig. 2B). In isolated rat islets 1 mM HC with adjusted NaCl also did

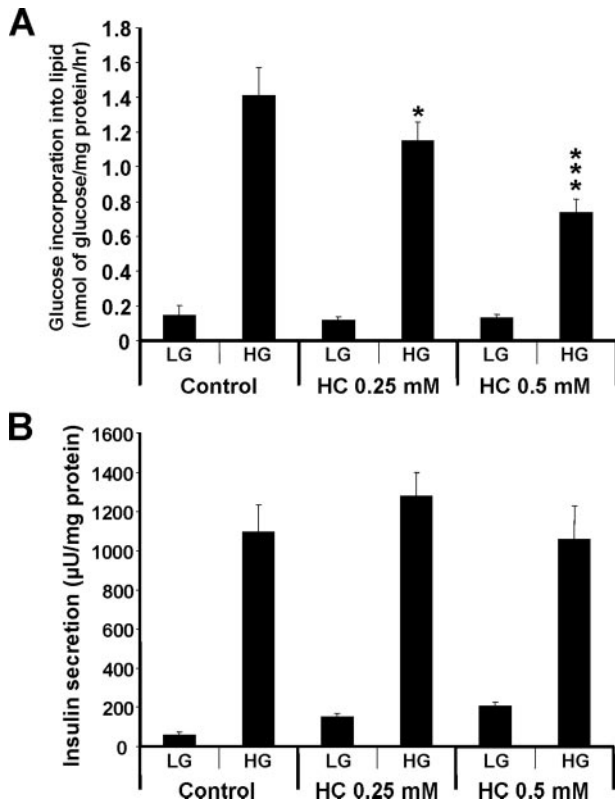


FIGURE 2. Lack of inhibitory effect of HC on GSIS from 832/13 cells when NaCl is balanced. For the experiments shown in this figure, we corrected for the large increase in NaCl caused by preparation of HC by standard methods (see "Experimental Procedures" for details). The effects of HC on (A) [14 C]glucose incorporation into lipid and (B) glucose-stimulated insulin secretion in 832/13 cells. For both panels, data represent the mean \pm S.E. for four independent experiments. *, $p < 0.05$; ***, $p < 0.001$ HG control versus HG plus HC. Low glucose (LG; 2.8 mM) and high glucose (HG; 16.7 mM) are shown.

not affect GSIS in either static incubation studies (Fig. 3A) or in perfused islets (Fig. 3B). We also investigated the effects of adding 130 mM NaCl to the KRB buffer. In islet perfusion experiments, this high salt buffer caused an increase in basal insulin secretion and inhibited GSIS in a pattern very similar to the effects of HC without salt balancing (Fig. 3C). In summary, these studies demonstrate that the CL inhibitor HC is not an inhibitor of GSIS when NaCl concentration is appropriately controlled.

A CL-specific siRNA Adenovirus Reduces Cytosolic Oxaloacetate, Malonyl-CoA Levels, and [14 C]Glucose Incorporation into Lipid—To further investigate the effects of CL suppression by an independent mechanism, and to allow more detailed metabolic studies and experiments in primary rat islets, we constructed recombinant adenoviruses containing two independent siRNA sequences specific for CL (Ad-siCL2 and Ad-siCL3). Treatment of 832/13 cells with Ad-siCL3 reduced CL protein levels by 75 ± 4 and $73 \pm 8\%$ at low or high glucose relative to cells treated with Ad-siControl (Fig. 4A). Furthermore, Ad-siCL3 treatment of 832/13 cells reduced CL activity by $85 \pm 4\%$ (Fig. 4B). Similar results were obtained for CL protein levels and enzymatic activity with Ad-siCL2 (data not shown). Because CL cleaves cytosolic citrate to yield acetyl-CoA and oxaloacetate, we measured cytosolic oxaloacetate and total

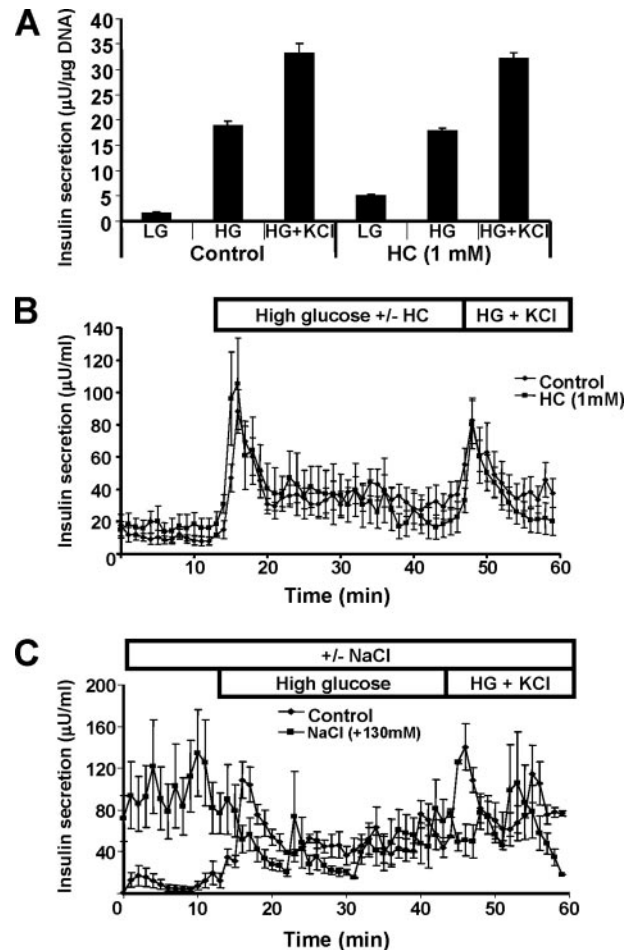


FIGURE 3. Effects of HC on GSIS in isolated rat islets. A, the effects of HC on GSIS in static incubation studies of rat islets after balancing for the additional NaCl added during HC preparation ($n = 6$). B, the effects of 1 mM HC on perfused rat islets after balancing for excess NaCl ($n = 6$). C, effects of adding 130 mM NaCl on GSIS in perfused rat islets ($n = 6$). Low glucose (LG; 2.8 mM), high glucose (HG; 16.7 mM) or high glucose plus KCl (HG+KCl; 16.7 mM glucose and 30 mM KCl) are shown.

malonyl-CoA levels to demonstrate anticipated metabolic effects of CL knockdown. Ad-siCL3 treatment of 832/13 cells reduced cytosolic oxaloacetate levels by $52 \pm 7\%$ (Fig. 5A) and total cell malonyl-CoA levels by $83 \pm 4\%$ (Fig. 5B) compared with Ad-siControl-treated cells.

CL is a critical enzyme for glucose-induced increases in lipogenesis. Accordingly, treatment of 832/13 cells with Ad-siCL3 reduced [14 C]glucose incorporation into lipids by $43 \pm 4\%$ (Fig. 5C); similar results were obtained with the independent CL-specific adenovirus, Ad-siCL2 (data not shown).

CL Suppression Does Not Affect GSIS—Having demonstrated that suppression of CL expression results in anticipated decreases in oxaloacetate and malonyl-CoA levels, as well as suppression of glucose incorporation into lipids, we next examined the effects of CL suppression on GSIS in 832/13 cells. As shown in Fig. 6, treatment of 832/13 cells with Ad-siCL3 caused a small but significant increase in basal insulin secretion, but did not alter the strong response to stimulatory glucose relative to Ad-siControl-treated cells (similar results were obtained with Ad-siCL2, data not shown).

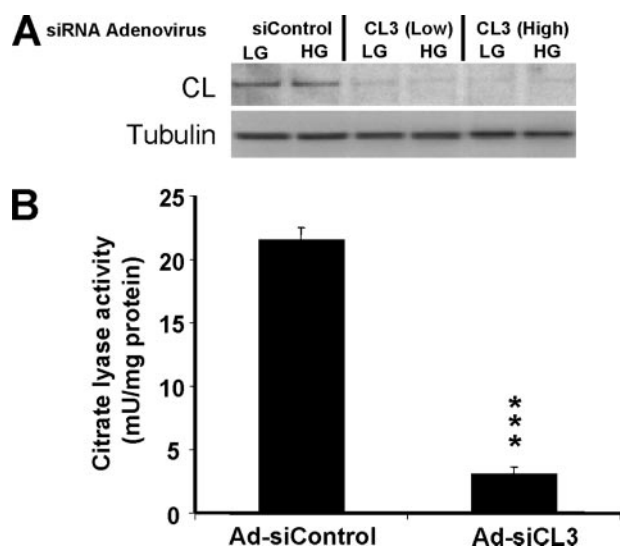


FIGURE 4. The effects of Ad-siCL3 on CL protein levels and enzymatic activity in 832/13 cells. 832/13 cells were treated with Ad-siCL3 or Ad-siControl. *A*, the effects of Ad-siCL3 on CL protein levels. Protein samples were harvested at both low glucose (LG; 2.8 mM) and high glucose (HG; 16.7 mM) levels. A representative immunoblot is shown from among three independent experiments. *B*, the effects of Ad-siCL3 on CL enzymatic activity. ***, $p < 0.001$ Ad-siControl versus Ad-siCL3. For panel *B*, data represent the mean \pm S.E. for four independent experiments.

CL Suppression Does Not Affect Pyruvate Cycling in 832/13 Cells—Our prior studies have suggested that pyruvate cycling is important for controlling insulin release, but the relative roles of the pyruvate/malate, pyruvate/citrate, and pyruvate/isocitrate pathways is still under investigation. CL would play a key role in the pyruvate/citrate cycling pathway. To investigate the contribution of CL to pyruvate cycling activity measured by ^{13}C NMR, we treated 832/13 cells with the CL3 siRNA duplex, which resulted in an $81 \pm 4\%$ suppression of CL mRNA levels. Consistent with our findings with recombinant adenoviruses summarized above, CL siRNA duplex transfection had no effect on GSIS (Fig. 7A) and also did not affect pyruvate cycling activity at basal or stimulatory glucose in 832/13 cells (Fig. 7B).

A FAS-specific siRNA Adenovirus Inhibits [^{14}C]Glucose Incorporation into Lipid and Does Not Affect GSIS—To further investigate the role of glucose incorporation into lipid in control of insulin secretion we constructed a recombinant adenovirus containing an siRNA sequence specific to FAS (Ad-siFAS). Treatment of 832/13 cells with Ad-siFAS reduced FAS mRNA levels by $82 \pm 2\%$ and protein levels by $61 \pm 7\%$ relative to Ad-siControl-treated cells (Fig. 8, *A* and *B*). Ad-siFAS-treated cells also exhibited a $61 \pm 7\%$ decrease in [^{14}C]glucose incorporation into lipid (Fig. 8C). Despite this clear inhibition of lipogenesis, Ad-siFAS treatment had no effect on GSIS (Fig. 8D).

Suppression of CL or FAS Expression Has no Effect on GSIS in Isolated Rat Islets—Finally, we tested whether the lack of effect of CL or FAS suppression on GSIS in 832/13 cells also pertains to primary rat islets. Treatment of rat islets with Ad-siCL3 reduced CL mRNA levels by $65 \pm 4\%$ and protein levels by $45 \pm 7\%$ (Fig. 9, *A* and *B*). This maneuver had no effect on insulin secretion at basal or stimulatory glucose compared with Ad-siControl-treated islets (Fig. 9C). Treatment of rat islets with

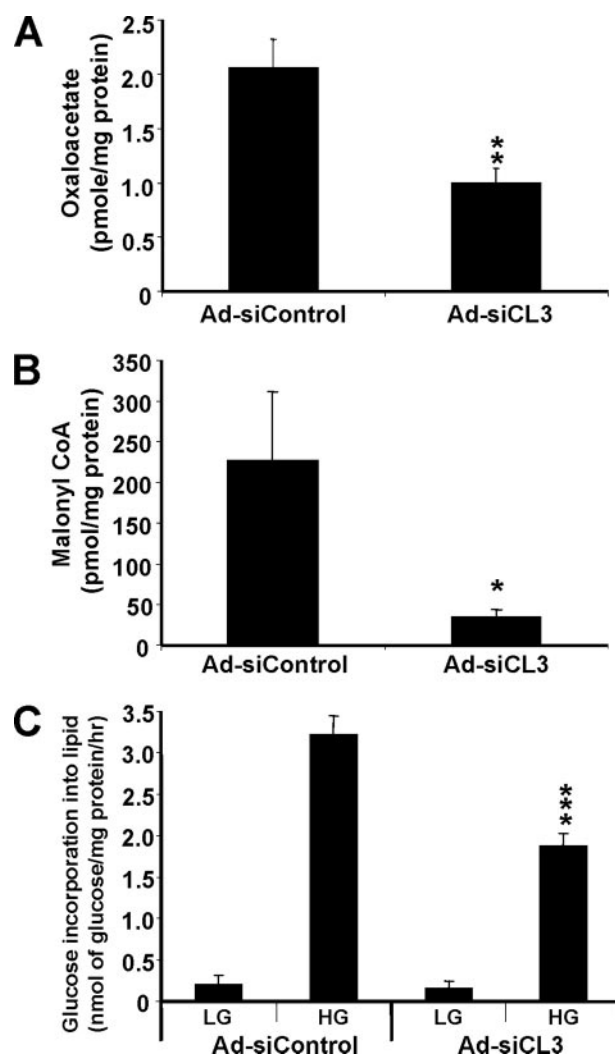


FIGURE 5. Metabolic impact of siRNA-mediated suppression of CL expression in 832/13 cells. 832/13 cells were treated with Ad-siCL3 or Ad-siControl and harvested for various metabolic assays. *A*, cytosolic oxaloacetate levels; *B*, malonyl-CoA levels. *, $p < 0.05$; **, $p < 0.01$ Ad-siControl versus Ad-siCL3. *C*, the effects of Ad-siCL3 on [^{14}C]glucose incorporation into lipid. For all panels, data represent the mean \pm S.E. for four independent experiments. ***, $p < 0.001$ Ad-siControl HG versus Ad-siCL3 HG.

Ad-siFAS reduced FAS mRNA levels by $52 \pm 3\%$ (Fig. 10A). Again, GSIS was not altered in islets treated with Ad-siFAS compared with Ad-siControl-treated islets (Fig. 10B).

DISCUSSION

The currently accepted pathway for GSIS involves an elevation in the ATP:ADP ratio, closure of ATP-sensitive K^+ channels (K_{ATP} channels), elevation of cytosolic Ca^{2+} levels, and activation of insulin granule exocytosis. However, it is clear from numerous studies that glucose retains an ability to stimulate insulin secretion even when K_{ATP} channels are rendered unresponsive, strongly implying that metabolic signals other than the ATP:ADP ratio are important for GSIS. Several products of mitochondrial metabolism of glucose have been suggested as complementary mediators of GSIS, including glutamate, malonyl-CoA, LC-CoA, α -KG, GTP, and NADPH (8–16, 38, 39). In the current study, we investigated the role of CL in GSIS because it is a key source of cytosolic acetyl-CoA for mal-

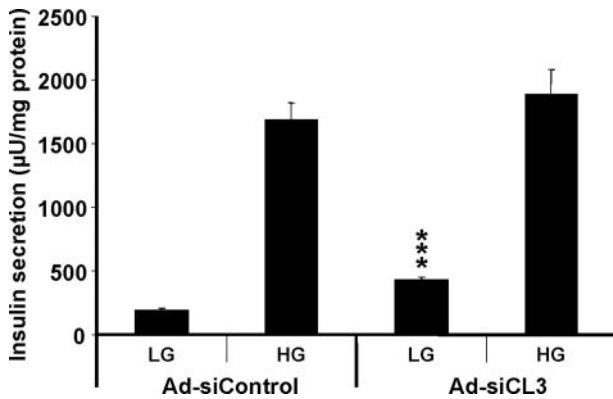


FIGURE 6. siRNA-mediated suppression of CL expression in 832/13 cells has no effect on GSIS. 832/13 cells were treated with Ad-siCL3 or Ad-siControl and assayed for GSIS by static incubation. Data represent the mean \pm S.E. for five independent experiments. Suppression of CL expression did cause a modest but significant (***) increase in insulin secretion at basal glucose (3 mM) compared with Ad-siControl cells, as indicated. LG, low glucose.

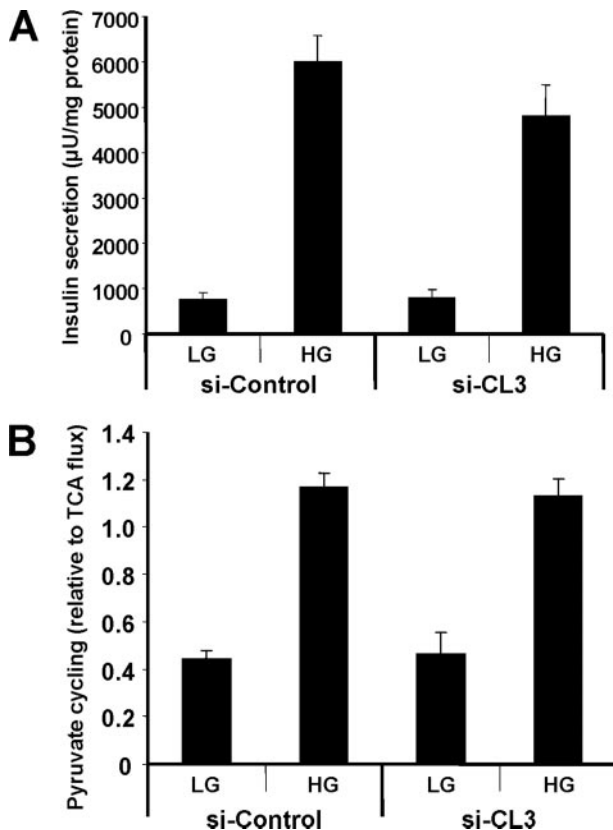


FIGURE 7. The effects of an siRNA duplex against CL (siCL3) on insulin secretion and pyruvate cycling. 832/13 cells were transfected with siCL3 or a control siRNA duplex, cultured for 72 h, and then incubated with [^{13}C]glucose for 4 h. A, insulin secretion; B, pyruvate cycling activity. Data in both panels represent the mean \pm S.E. for four independent experiments. HG, high glucose (16.7 mM); LG, low glucose (2.8 mM).

onyl-CoA and LC-CoA generation and because it could play an important role in pyruvate cycling via the pyruvate/citrate pathway. To further investigate the role of *de novo* lipogenesis in β -cell stimulus/secretion coupling, we have also performed studies on the effects of manipulation of FAS expression. The overarching and consistent message from all of the experiments described herein, involving both pharmacologic and siRNA-

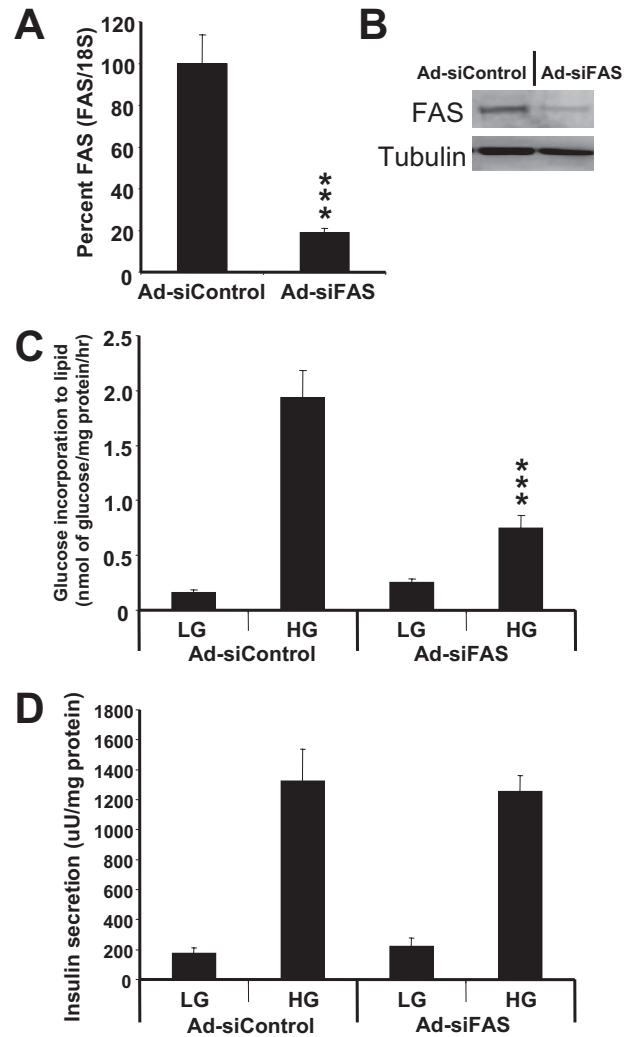


FIGURE 8. The effects of Ad-siFAS on FAS expression, glucose incorporation into lipids, and GSIS in 832/13 cells. 832/13 cells were treated with Ad-siFAS or Ad-siControl. A, FAS mRNA levels; B, FAS protein levels, representative immunoblot from four independent experiments; C, incorporation of [^{14}C]glucose into lipids; D, GSIS. ***, $p < 0.001$ Ad-siControl HG versus Ad-siFAS HG. For panels A, C, and D, data represent the mean \pm S.E. for five to six independent experiments. HG, high glucose (16.7 mM); LG, low glucose (2.8 mM).

mediated suppression of CL, as well as siRNA-mediated suppression of FAS expression in clonal β -cells and primary rat islets, is that pyruvate/citrate cycling and *de novo* lipogenesis do not play important roles in control of GSIS.

The current findings are consistent with previous studies from our laboratory and others demonstrating that prevention of the glucose-induced rise in malonyl-CoA levels by overexpression of malonyl-CoA decarboxylase has no impact on GSIS (27–29). Also consistent with the current findings are reports that treatment of β -cells with the LC-CoA synthetase inhibitor triacsin C has no effect on GSIS (27–29). It remains possible, however, that malonyl-CoA and LC-CoA could play a role in fatty acid-induced potentiation of GSIS, because experiments with triacsin C and malonyl-CoA decarboxylase overexpression diminished this action of free fatty acid in β -cell lines and rat islets in some studies (29, 40), although not in others (28). Adding to the complexity are recent findings suggesting that

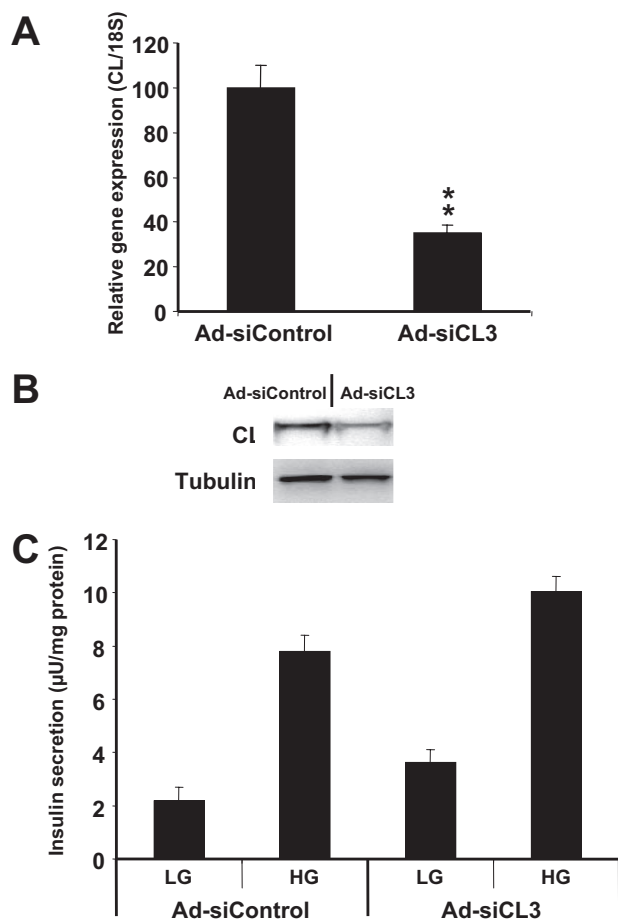


FIGURE 9. The effects of Ad-siCL3 on CL expression and GSIS in isolated rat islets. Rat islets were treated with Ad-siCL3 or Ad-siControl. *A*, CL mRNA levels; *B*, CL protein levels, representative immunoblot from three independent experiments; *C*, GSIS. **, $p < 0.01$ Ad-siControl versus Ad-siCL3. Data in panels *A* and *C* represent the mean \pm S.E. for five independent experiments. *HG*, high glucose (16.7 mM); *LG*, low glucose (2.8 mM).

fatty acids potentiate GSIS by a receptor-based mechanism involving the G protein-coupled receptor GPR40, although it is also suggested that part of the potentiating effect may be due to a lipid metabolism-generated signal (41–44). Overall, whereas the mechanism of lipid-mediated potentiation of GSIS remains to be clarified, our findings provide strong evidence against a direct role of CL, malonyl-CoA, or FAS-catalyzed *de novo* lipogenesis in regulation of GSIS when studied in the absence of other potentiators. We do acknowledge the caveat that CL and FAS were not completely suppressed by the siRNA methods employed in this study, and that the residual lipogenic flux could have enabled the glucose response in some way. However, the results certainly establish that there is no linear relationship between lipogenic flux and GSIS.

We show here that inhibition of CL with HC inhibits [U - ^{14}C]glucose incorporation into lipid but does not inhibit GSIS in either 832/13 cells or in isolated rat islets. Some laboratories have reported that HC inhibits insulin release from purified rat β -cells or the perfused rat pancreas (37, 45), whereas other groups have reported no effect of HC on insulin secretion (46). The method of preparation of HC in the prior studies was not elaborated. If prepared by an acid-solubilization/base neutralization method as described herein, it is likely

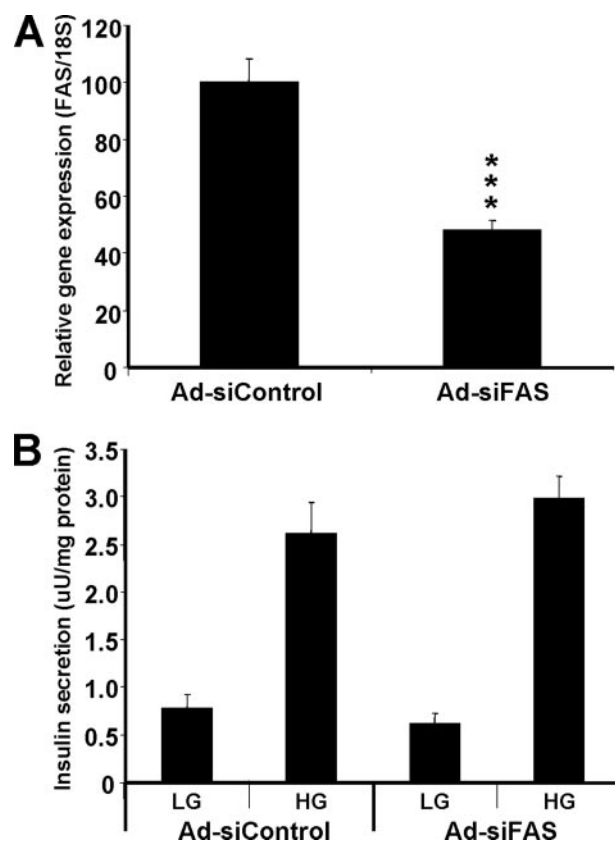


FIGURE 10. The effects of Ad-siFAS on FAS expression and GSIS in isolated rat islets. Rat islets were treated with Ad-siFAS or Ad-siControl. *A*, FAS mRNA levels; *B*, GSIS. ***, $p < 0.01$ Ad-siControl versus Ad-siFAS. Data represent the mean \pm S.E. for five independent experiments. *HG*, high glucose (16.7 mM); *LG*, low glucose (2.8 mM).

that any inhibitory effect on GSIS is due to the accumulation of high levels of NaCl, as demonstrated in Figs. 1–3 of the current study.

An inhibitor of FAS, cerulenin, has been shown to inhibit GSIS in isolated rat islets (47–50). One possible explanation for the apparent discrepancy with the current findings is that cerulenin, a natural product obtained from the fungus *Cephalosporium caerulens*, has been shown to have effects in addition to FAS inhibition, including inhibition of protein palmitoylation (51). The compound also inhibits hydroxymethylglutaryl-CoA synthetase activity, resulting in reduced biosynthesis of cholesterol and other sterols (52). Such effects would not be anticipated when FAS is inhibited by the RNA interference technology used in the current study.

In pursuit of the metabolic mechanism of GSIS, the findings of the current study shift focus away from malonyl-CoA, LC-CoA, and pyruvate/citrate cycling as key mediators of glucose sensing. Instead, focus is recast on the potential roles of the pyruvate/isocitrate or pyruvate/malate cycling pathways (15, 16, 53). In support of a key role of pyruvate/isocitrate cycling, recent studies from our group have demonstrated that pharmacologic or siRNA-mediated suppression of the mitochondrial citrate/isocitrate carrier, or siRNA-mediated suppression of cytosolic, NADP⁺-dependent ICDC results in strong impairment of GSIS (15, 16). Possible second messengers of a pyruvate/isocitrate pathway include NADPH, α -KG, or GTP.

NADPH has emerged as a candidate via studies demonstrating good correlations between NADPH:NADP⁺ levels and GSIS (11, 12, 14, 15, 20), and in studies demonstrating stimulation of insulin secretion by addition of NADPH to permeabilized β -cells (11). Other work has suggested that the differential responses of islets from BTBR and B6 mouse strains to α -ketoisocaproate can be equalized by addition of α -KG, suggesting a direct stimulatory effect of this intermediate (the product of the ICDc reaction) or a downstream metabolite (39). Finally, a recent study has demonstrated that further metabolism of α -KG via succinyl-CoA synthetase may contribute to control of insulin secretion via production of GTP (38). Other work implicates pyruvate/malate cycling and the cytosolic NADP⁺-linked malic enzyme (53), because siRNA-mediated suppression of cytosolic, but not mitochondrial malic enzyme activity was reported to cause impairment of GSIS in 832/13 cells. However, no firm conclusions about a role for malic enzyme in GSIS can be made until the findings are confirmed in primary islet preparations. Such confirmatory data has been provided for all of the enzymes and carriers (citrate/isocitrate carrier, ICDc, CL, FAS, and PC) analyzed in our previous (14–16) and the current studies.

In conclusion, the studies described herein have investigated the role of CL and FAS in control of GSIS. Whereas we confirm that these enzymes play important roles in regulation of *de novo* lipogenesis from glucose in β -cells, we find no evidence that they play a regulatory role in GSIS. Instead, our studies and those of others now focus attention on pyruvate/isocitrate and pyruvate/malate pathways as potential cycles for generation of key stimulus/secretion coupling intermediates for GSIS.

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