

Chronic Suppression of Acetyl-CoA Carboxylase 1 in β -Cells Impairs Insulin Secretion via Inhibition of Glucose Rather Than Lipid Metabolism*

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Sarah M. Ronnebaum[‡], Jamie W. Joseph[‡], Olga Ilkayeva[‡], Shawn C. Burgess[§], Danhong Lu[‡], Thomas C. Becker[‡], A. Dean Sherry[§], and Christopher B. Newgard^{‡1}

From the [‡]Sarah W. Stedman Nutrition and Metabolism Center and Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27704 and [§]Departments of Radiology and Pharmacology, Mary Nell and Ralph B. Rogers Magnetic Resonance Center and Advanced Imaging Research Center, University of Texas Southwestern Medical Center, Dallas, Texas 75235

Acetyl-CoA carboxylase 1 (ACC1) currently is being investigated as a target for treatment of obesity-associated dyslipidemia and insulin resistance. To investigate the effects of ACC1 inhibition on insulin secretion, three small interfering RNA (siRNA) duplexes targeting ACC1 (siACC1) were transfected into the INS-1-derived cell line, 832/13; the most efficacious duplex was also cloned into an adenovirus and used to transduce isolated rat islets. Delivery of the siACC1 duplexes decreased ACC1 mRNA by 60–80% in 832/13 cells and islets and enzyme activity by 46% compared with cells treated with a non-targeted siRNA. Delivery of siACC1 decreased glucose-stimulated insulin secretion (GSIS) by 70% in 832/13 cells and by 33% in islets. Surprisingly, siACC1 treatment decreased glucose oxidation by 49%, and the ATP:ADP ratio by 52%, accompanied by clear decreases in pyruvate cycling activity and tricarboxylic acid cycle intermediates. Exposure of siACC1-treated cells to the pyruvate cycling substrate dimethylmalate restored GSIS to normal without recovery of the depressed ATP:ADP ratio. In siACC1-treated cells, glucokinase protein levels were decreased by 25%, which correlated with a 36% decrease in glycogen synthesis and a 33% decrease in glycolytic flux. Furthermore, acute addition of the ACC1 inhibitor 5-(tetradecyloxy)-2-furoic acid (TOFA) to β -cells suppressed [¹⁴C]glucose incorporation into lipids but had no effect on GSIS, whereas chronic TOFA administration suppressed GSIS and glucose metabolism. In sum, chronic, but not acute, suppression of ACC1 activity impairs GSIS via inhibition of glucose rather than lipid metabolism. These findings raise concerns about the use of ACC inhibitors for diabetes therapy.

The key lipogenic enzyme, ACC1, has become a target of interest for obesity and diabetes therapies, as its inhibition is predicted to decrease *de novo* lipogenesis and possibly help

remedy the systemic and tissue hyperlipidemia associated with these disorders. However, if drugs that target ACC² are to be used safely, two important concerns must be addressed.

First, current ACC inhibitors do not discriminate between the ACC1 and ACC2 isoforms. The former enzyme plays a prominent role in synthesis of new fatty acids from glucose and other fuels, whereas the latter is thought to participate in regulation of fatty acid oxidation via production of a mitochondria-localized pool of malonyl-CoA that regulates carnitine palmitoyltransferase 1 (CPT1). Consistent with these ideas, whole body knock-out of ACC1 in mice is embryonic lethal (1), and liver-specific knock-out impairs hepatic triglyceride synthesis without affecting glucose homeostasis (2). In contrast, whole body knock-out of ACC2 results in mice that are lean and protected against diet-induced insulin resistance (3, 4). The consequences of chronic and simultaneous inhibition of both forms of ACC remain to be fully described.

Second, the role of ACC1 in regulation of insulin secretion is unclear. Stable overexpression of ACC1-specific antisense constructs has been reported to result in impaired GSIS (5), but the mechanism by which this occurs has not been elucidated. Conversely, treatment of β -cells with the liver X receptor agonist T0901317 increases expression of a number of lipogenic genes, including ACC1, in concert with an increase in GSIS (6). In contrast, others have reported that stimulation of 5'-AMPK (AMP-dependent kinase) activity by molecular or pharmacologic methods causes phosphorylation and inhibition of ACC but with no effect on GSIS (7).

GSIS occurs in two phases. The first phase is believed to be catalyzed by an acute rise in the ATP:ADP ratio brought about by mitochondrial glucose oxidation, ATP-dependent potassium (K_{ATP}) channel closure, membrane depolarization, and calcium ion influx via voltage-gated calcium channels. The second, prolonged phase of insulin secretion has been associated with a variety of metabolic processes, including glutamate

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¹ To whom correspondence should be addressed: Sarah W. Stedman Nutrition and Metabolism Ctr., Duke Independence Park Facility, 4321 Medical Park Dr., Suite 200, Durham, NC 27704. Fax: 919-477-0632; E-mail: newgard002@mc.duke.edu.

² The abbreviations used are: ACC, acetyl-CoA carboxylase; pACC1, phosphorylated ACC1; Ad-siControl, control adenovirus; Ad-siACC1, adenovirus expressing siRNA targeting ACC1; CPT1, carnitine palmitoyltransferase 1; DMM, dimethylmalate; GC/MS, gas chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; GK, glucokinase; Glut2, glucose transporter 2; GSIS, glucose-stimulated insulin secretion; PC, pyruvate carboxylase; siRNA, small interfering RNA; TOFA, 5-(tetradecyloxy)-2-furoic acid; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

metabolism (8), pyruvate cycling (9), the NADH shuttle system (10), *de novo* lipogenesis/long-chain acyl-CoAs (11, 12), and NADPH production (13–15). Fatty acids have complicated effects on β -cells, in that acute administration potentiates GSIS, apparently in part via activation of the G protein-coupled receptor GPR40 (16, 17), whereas chronic exposure (>24 h) inhibits insulin secretion, possibly because of impaired regulation of pyruvate cycling (18), increased oxidative stress (19), and/or depletion of insulin reserves via stimulation of basal insulin secretion (20). Less clear is the role of endogenously produced long-chain acyl-CoAs in the regulation of insulin secretion (21), because expression of malonyl-CoA decarboxylase in β -cells decreases *de novo* lipogenesis with no effect on GSIS in the absence of fatty acids (22–24). In light of all of these considerations, a better understanding of the potential role of ACC1 in regulation of GSIS is required. The goal of the present study was to investigate the effects of pharmacologic and molecular suppression of ACC1 activity on insulin secretion and metabolism in both insulinoma cell lines and primary rat islets.

We found that chronic, but not acute, suppression of ACC1 expression or activity resulted in a significant impairment of GSIS. This effect was linked to an unexpected decrease in glucokinase (GK) protein expression, as well as inhibition of glycolytic flux, glucose oxidation, and ATP production, rather than to a decrease in lipogenic flux. These findings provide a cautionary note for those pursuing ACC1 as a therapeutic target for diabetes and obesity.

EXPERIMENTAL PROCEDURES

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

Cell Lines—Two clonal cell lines that exhibit robust GSIS, 832/3 and 832/13 (25), were derived from the rat insulinoma cell line INS-1 (26). Briefly, parental INS-1 cells were transfected with a plasmid containing a cytomegalovirus-human insulin expression construct and a neomycin selection gene followed by isolation of discrete neomycin-resistant cell colonies (subclones) (25). Cells were cultured as described previously (25, 26).

Real Time PCR—RNA was isolated from 832/13 cells and tissues as described (15). Measurements were performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The SYBR primer sequences were: ACC1 forward, GCC ATC CGG TTT GTT GTC A; ACC1 reverse, GGA TAC CTG CAG TTT GAG CCA; ACC2 forward, AAG TCA TCT CCT GCT TTG CC; ACC2 reverse, TGC AAA CTC ATC TCT CGC TCT; GK forward, TCG TGT CAC AAG TGG AGA GC; GK reverse, GCG ATT TAT GAC CCC AGC TA; HK1 forward, ACG ACA CCC CAG AGA ACA TC; HK1 reverse, GAA GTC TCC CAG GCA GTC AG. All other primers were Taqman Assays on Demand (Applied Biosystems).

siRNA Duplex-mediated Gene Suppression—Small interfering RNA (siRNA) duplexes (Ambion, Austin, TX, and Integrated DNA Technologies, Coralville, IA) were transfected into INS-1-derived cell lines by exposure of cells to 50 nM duplexes for 24 h in the presence of Dharmafect Reagent 1 (Dharmacon, Lafayette, CO). Duplexes were targeted to 19–21-bp regions

of the rat ACC1 cDNA sequence (GenBankTM accession number NM_022193) beginning at nucleotides 259, 354, and 5745 relative to the start codon. A duplex with no known sequence homology or biological effect (siControl) was used as a control (15). Following a 24-h transfection period, cells were cultured for an additional 48 h prior to assays.

Recombinant Adenovirus Construction—Adenoviruses containing siRNA sequences specific for ACC1 (Ad-siACC1) or siControl (Ad-siControl) were constructed using vectors EH006 and pJM17 as described (27, 28). The ACC1 siRNA target sequence was GAA GGT TCT TAT CGC CAA CAA, which corresponds to ACC1 siRNA duplex 354. Viruses were purified using a BD Biosciences Adeno-X purification kit (Clontech, Palo Alto, CA), and virus titer was calculated by end-point dilution assay according to the manufacturer's guidelines.

Immunoblot Analysis—Lysates of siRNA-transfected cells were prepared in m-PER lysis buffer (Pierce) containing Halt phosphatase inhibitor mixture (Pierce) and phosphatase inhibitor mixtures 1 and 2 (Sigma). Total protein concentrations were determined using the BCA protein assay (Pierce). Proteins were loaded onto NuPage 3–8% Tris acetate gels or NuPage 4–12% bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked with 2% ECL advance blocking reagent (GE Healthcare). Phosphorylated ACC1 (Cell Signaling, Danvers, MA), Glut2 (Chemicon, Temecula, CA), GK (Abcam, Cambridge, MA), and γ -tubulin (Sigma) antibodies were diluted according to the manufacturer's guidelines in Tris-buffered saline, 1% polyvinylpyrrolidone, and secondary antibodies were diluted 1:10,000 (GE Healthcare) in Tris-buffered saline. ACC1, pyruvate carboxylase, and propionyl-CoA carboxylase were measured using horseradish peroxidase-conjugated streptavidin-peroxidase polymer (Sigma) and quantified as described previously (29).

Insulin Secretion and Content Assays—Insulin secretion and insulin content measurements were performed in INS-1-derived cell lines as described previously (29). For GSIS assays in islets, pancreatic islets were harvested from male Sprague-Dawley rats weighing ~250 g, as described previously (30, 31). Islets were transduced with either Ad-siControl or Ad-siACC1 for 48 h at 4×10^7 plaque-forming units/islet. Islets were cultured for an additional 24 h before GSIS assays were performed as described previously (15), and data were normalized to protein content.

ACC1 Activity Assay—ACC1 enzymatic activity was measured in 832/13 cell extracts by the method of Kowluru *et al.* (32).

Glucose Incorporation into Lipids—[U-¹⁴C]Glucose (GE Healthcare) incorporation into lipids was measured as described previously (22).

Glucose Oxidation and Oxygen Consumption—[U-¹⁴C]Glucose oxidation in 832/13 cells (15) and oxygen consumption (18) were measured as described previously. [U-¹⁴C]Glucose oxidation in dispersed islets was measured on groups of 75,000 cells incubated with 16.7 mM glucose at a specific activity of 0.5 Ci/mol. Cells were aliquoted in [U-¹⁴C]glucose medium in the trap apparatus and shaken in a 37 °C water bath for 1 h. Samples

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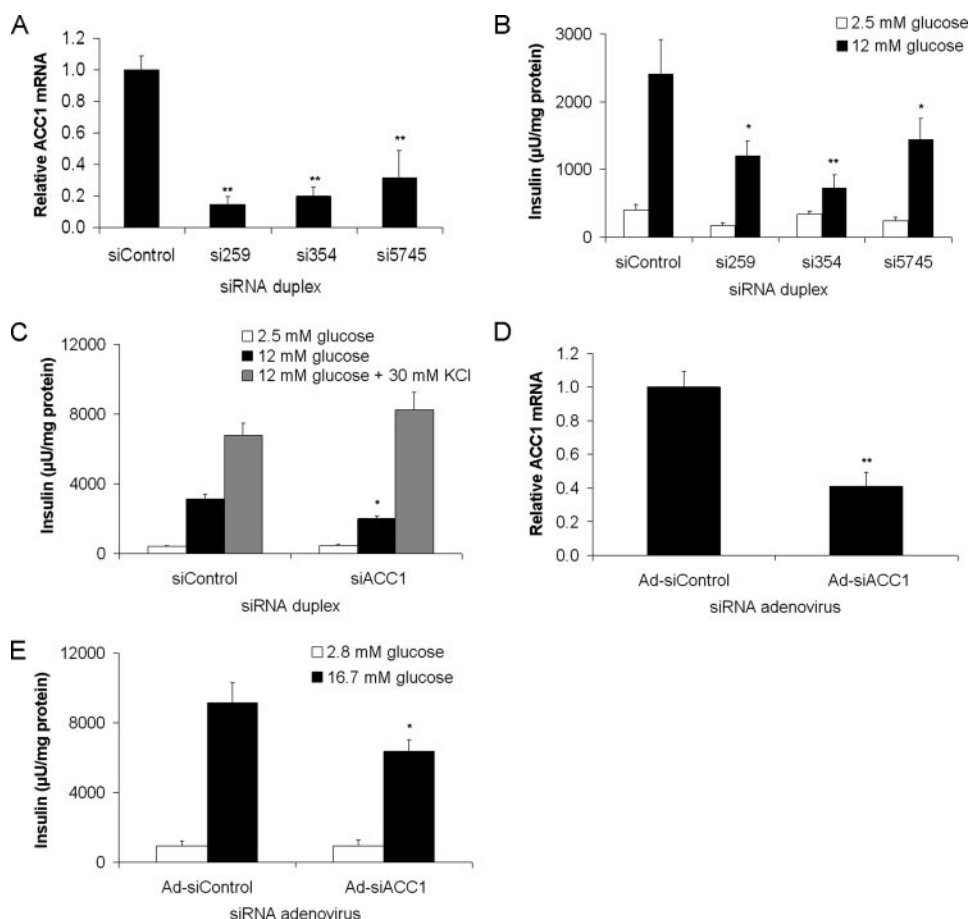


FIGURE 1. Suppression of ACC1 expression decreases insulin secretion in 832/13 cells and islets. 832/13 cells were transfected with one of three siRNA duplexes targeting ACC1 or a scrambled sequence (siControl) for 24 h; following an additional 48 h of culture, ACC1 mRNA (A) and insulin secretion at 2.5 or 12 mM glucose (B) were measured. C, insulin secretion was measured in 832/13 cells transfected with siControl or si354 (siACC1) in the absence or presence of 30 mM KCl. Rat pancreatic islets were transfected for 48 h with Ad-siControl or Ad-siACC1 (target 354); following 24 h of additional culture, ACC1 mRNA (D) and insulin secretion at 2.8 and 16.7 mM glucose (E) were measured. Results represent mean \pm S.E. of three independent experiments, *, $p < 0.05$, **, $p < 0.001$, compared with siControl.

were then acidified with trichloroacetic acid and oxidation rates calculated as described (15).

Profiling of Intermediary Metabolites—Cells were cultured in 15-cm dishes and incubated for 2 h in 2.5 or 12 mM glucose. Following collection of media samples from these cells for GSIS measurement, cell lysates were prepared for measurement of seven organic acid species by GC/MS as described previously (29). For profiling of acylcarnitine species, cells were cultured for 24 h prior to assay in medium containing 1 mM L-carnitine. Lysates were prepared and processed for tandem mass spectrometry (MS/MS) analysis as described (29).

^{13}C NMR Mass Isotopomer Analysis—Pyruvate cycling was measured via ^{13}C NMR isotopomer analysis as described previously (9) in cells exposed to 2.5 and 12 mM $[\text{U}-^{13}\text{C}]$ glucose.

ATP and ADP Determination—832/13 cells were cultured in 6-well plates and processed as described for GSIS studies. Following 2 h of incubation at 2.5 or 12 mM glucose, cells were scraped in incubation buffer on ice and centrifuged, and the remaining cell pellet was snap-frozen in a dry ice-ethanol bath. ATP and ADP were measured as described previously (33, 34).

Fatty Acid Oxidation— $[1-^{14}\text{C}]$ Palmitate oxidation was measured as described (22, 23).

Glucose Usage— $[5-^3\text{H}]$ Glucose (GE Healthcare) usage was measured as described (35).

GK Activity Assay—GK activity was determined from the difference in glucose phosphorylating activity at 0.5 and 100 mM glucose, representing low K_m and total hexokinase activity, respectively, using a previously described method (36).

Glycogen Synthesis—Cells were incubated in buffer containing $[\text{U}-^{14}\text{C}]$ glucose as described for glucose oxidation assays. Buffer was removed, and cells were lysed in saturated KOH, incubated at 95 °C for 10 min, and then cooled. Ethanol was added to precipitate glycogen, and samples were incubated overnight at -20°C . Samples were centrifuged, and the pellet was washed, dried overnight, resuspended in water, heated, and counted using liquid scintillation.

Pharmacologic ACC Inhibition—5-(Tetradecyloxy)-2-furoic acid (TOFA) was resuspended in DMSO at a concentration of 10 mM. 832/13 cells were treated with medium containing 10 μM TOFA or 0.1% DMSO as control, and islets were treated with medium containing 100 μM TOFA or 1% DMSO as control, for time periods specified under “Results” and in the Fig. 6 legend.

Statistical Analysis—Data were expressed as the mean \pm S.E. of at least three independent experiments performed in duplicate or triplicate. Statistical significance was determined using a two-sample equal variance Student’s *t* test for assays with two sample sets and using one-way analysis of variance with Bonferroni post-hoc analysis for experiments with multiple experimental groups. A *p* value of <0.05 was considered significant.

RESULTS

ACC1 and ACC2 Tissue Expression Pattern—Both ACC1 and ACC2 catalyze the carboxylation of acetyl-CoA to form malonyl-CoA; however, these enzymes have different tissue distribution. ACC1 and ACC2 mRNA were measured by real time PCR in the liver, gastrocnemius muscle, and islets from lean rats, as well as in the rat INS-1-derived 832/13 β -cell line. We found ACC1 and ACC2 to be expressed in liver at comparable levels, whereas ACC2 was the principle isoform in skeletal muscle. Islets and 832/13 cells expressed ACC1 mRNA and very little ACC2 mRNA, consistent with a previous report (5) (data not shown). Therefore, we chose to focus exclusively on the role of ACC1 in the pancreatic β -cell.

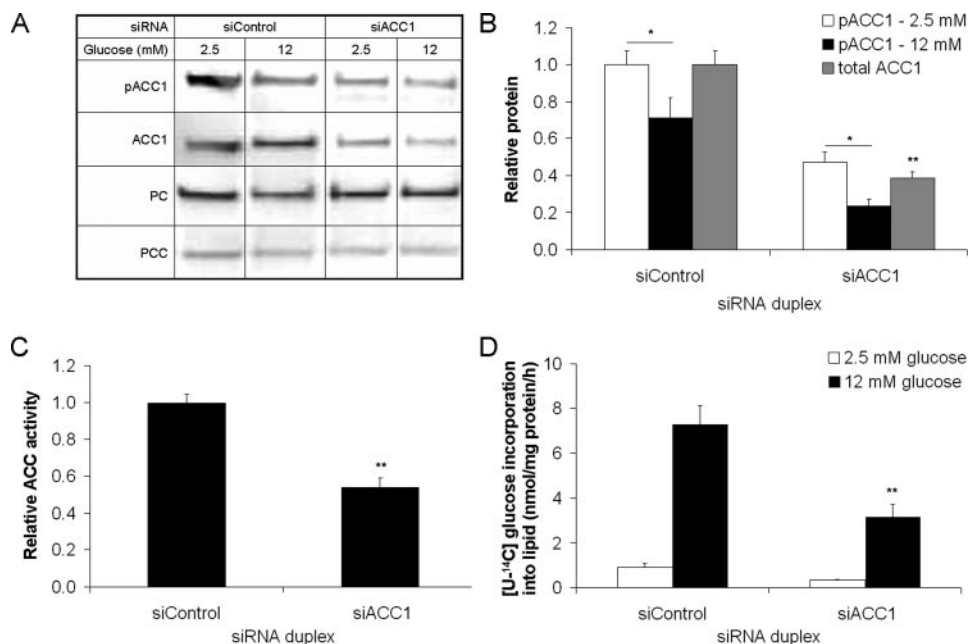


FIGURE 2. ACC1 siRNA treatment decreases ACC1 protein and enzyme activity. *A*, representative immunoblot of 832/13 cells transfected with siACC1 and blotted for phospho-ACC1 (pACC1), total ACC1 (ACC1), pyruvate carboxylase (PC), and propionyl-CoA carboxylase (PCC) as described under "Experimental Procedures." *B*, quantitative summary of pACC1 and total ACC1 levels normalized to PC levels. *C*, ACC1 enzymatic activity was measured in 832/13 cells. *D*, [U-¹⁴C]glucose incorporation into lipids was measured following a 2-h incubation in 2.5 or 12 mM glucose and was normalized to protein content and time. The results shown in *B*, *C*, and *D* represent mean \pm S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.001$.

ACC1 Suppression via siRNA Impairs GSIS in 832/13 Cells and Rat Islets—832/13 cells were transfected with one of three siRNA duplexes targeting distinct regions of the ACC1 transcript (si259, si354, and si5745), or with an siRNA sequence with no known gene homology (siControl). Following an additional 48 h of culture, ACC1 mRNA, ACC1 protein, and GSIS were measured. As shown in Fig. 1*A*, all three ACC1 siRNA duplexes significantly decreased ACC1 mRNA levels, with maximal suppression of $80 \pm 6\%$ by si354. Fig. 1*B* shows that the 832/13 cells used in these experiments exhibit robust GSIS, as stimulation of siControl-treated cells with 12 mM glucose caused a 6.1 ± 1.7 -fold increase in insulin secretion compared with secretion at 2.5 mM glucose. Insulin secretion in untreated or mock-treated cells was not significantly different from siControl-treated cells at either glucose concentration (data not shown). However, treatment of 832/13 cells with any of the three ACC1 siRNA duplexes decreased insulin secretion at 12 mM glucose by 40–70%, indicating that ACC1 may play a role in regulating GSIS. Similar results were obtained in an independent, highly glucose-responsive cell line, 832/3, in response to treatment with si354 (data not shown). 832/13 cells transfected with si354 had an insulin content similar to siControl-treated cells (53 ± 5 milliunits/mg protein compared with 49 ± 3 milliunits/mg protein). Finally, GSIS was measured in 832/13 cells in the presence or absence of 30 mM KCl. As shown in Fig. 1*C*, significant impairment of insulin secretion was observed in cells with suppressed ACC1 expression when stimulated with 12 mM glucose alone, but this suppression was not evident when cells were treated with 12 mM glucose plus 30 mM KCl, indicating that ACC1 suppression affects insulin secretion

through impairment of metabolic signaling and not by regulation of more distal events in the secretory pathway.

An adenovirus expressing a short hairpin RNA sequence corresponding to si354 was constructed (Ad-siACC1) and used to transduce isolated rat islets for GSIS studies. ACC1 mRNA was decreased by $59 \pm 8\%$ in islets treated with Ad-siACC1 compared with islets treated with the Ad-siControl adenovirus (Fig. 1*D*). Control islets demonstrated a robust 10-fold increase in insulin secretion when stimulated with 16.7 mM glucose (Fig. 1*E*). Ad-siACC1 treatment inhibited insulin secretion at high glucose by $33 \pm 9\%$ but had no effect on basal insulin secretion. Taken together, these data indicate that suppression of ACC1 expression impairs GSIS in insulinoma cells and primary rat islets.

ACC1 Protein and Activity Are Significantly Decreased with ACC1 siRNA Treatment

ACC1 is phosphorylated (pACC1) and inactivated at low glucose by AMP-dependent kinase (37), whereas in the presence of high glucose, dephosphorylation of ACC1 increases its activity (38). As shown in the representative immunoblot in Fig. 2*A*, in both control and siACC1-treated 832/13 cells, pACC1 levels are higher at 2.5 mM glucose than at 12 mM glucose. As shown in Fig. 2*B*, on average, siACC1 treatment caused a $61 \pm 4\%$ decrease in total ACC1 protein levels and a $53 \pm 6\%$ decrease in pACC1 levels at 2.5 mM glucose when normalized to pyruvate carboxylase (PC). As shown in Fig. 2*C*, siACC1 also decreased ACC enzymatic activity by $46 \pm 5\%$, indicating that siRNA-mediated suppression of ACC1 protein levels was proportional to effects on enzyme activity. Furthermore, as shown in Fig. 2*D*, [U-¹⁴C]glucose incorporation into lipids was suppressed by $57 \pm 12\%$ at 12 mM glucose in siACC1-treated compared with siControl-treated 832/13 cells, consistent with the decrease in ACC1 protein levels and enzymatic activity.

ACC1 siRNA Decreases Glucose Oxidation, Tricarboxylic Acid Cycle Intermediates, and Short-chain Acylcarnitines—To further address the metabolic effects of ACC1 suppression in 832/13 cells, we next measured glucose oxidation. Surprisingly, in siACC1-treated cells, glucose oxidation decreased by $56 \pm 8\%$ at 2.5 mM glucose and $49 \pm 10\%$ at 12 mM glucose compared with siControl-treated cells (Fig. 3*A*). Similarly, siACC1 treatment caused a complete ablation of the glucose-induced increase in oxygen consumption (1.5 ± 0.4 nmol/mg protein/min in siControl-treated cells compared with 1.0 ± 0.3 nmol/mg protein/min in siACC1-treated cells). These changes did not affect cell viability, as incorporation of [methyl-³H]thymidine into DNA and total cell number per well were

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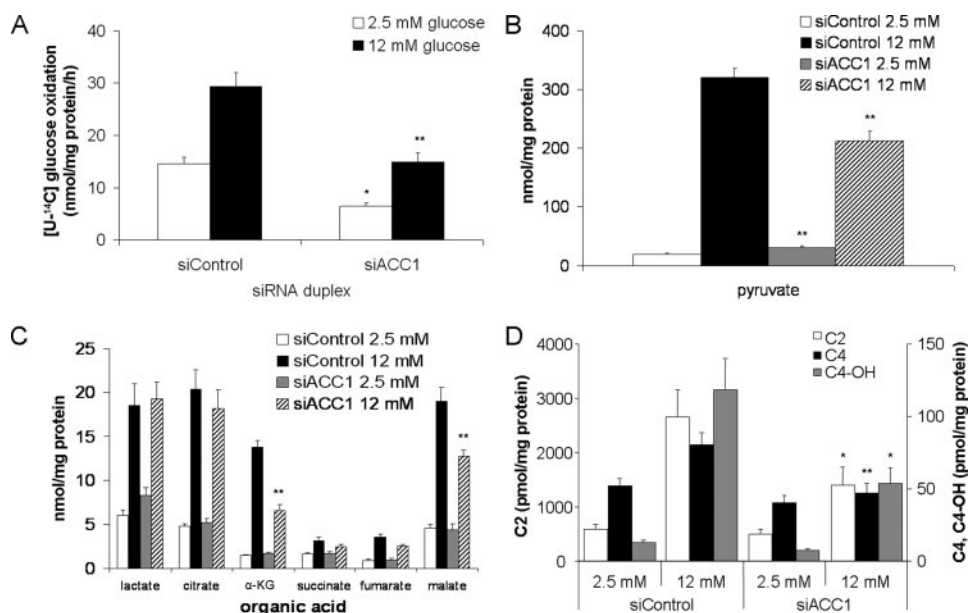


FIGURE 3. Suppression of ACC1 expression decreases glucose oxidation and levels of mitochondrial metabolites. A, [U-¹⁴C]glucose oxidation was measured at 2.5 and 12 mM glucose in 832/13 cells transfected with siACC1 or siControl duplexes. Pyruvate (B) and six other organic acids (C) were measured by GC/MS in cells incubated in 2.5 or 12 mM glucose. The results shown in A, B, and C represent the mean ± S.E. of four independent experiments. α-KG, α-ketoglutarate. D, C2 (acetyl), C4 (butyryl), and C4-OH (β-hydroxybutyryl) acylcarnitines were measured by MS/MS in cells incubated in 2.5 or 12 mM glucose. The results were normalized to protein content and represent the mean ± S.E. of three independent experiments, **p* < 0.01; ***p* < 0.001.

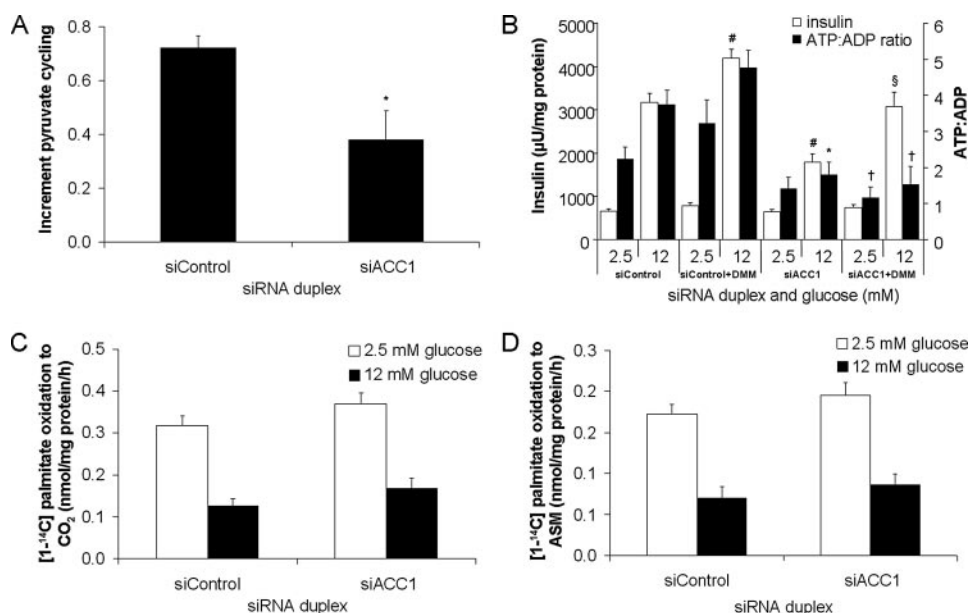


FIGURE 4. Suppression of ACC1 expression impairs the glucose-induced rise in pyruvate cycling and ATP:ADP ratio. A, the increment in pyruvate cycling activity caused by raising [U-¹³C]glucose levels from 2.5 to 12 mM in siACC1- and siControl-treated 832/13 cells was measured via ¹³C NMR mass isotopomer analysis. Results represent the mean ± S.E. of four independent experiments, **p* < 0.05. B, 832/13 cells were incubated in 2.5 or 12 mM glucose in the presence or absence of 10 mM DMM for 2 h followed by measurement of ATP, ADP, and insulin secretion. The ATP:ADP ratio was calculated and plotted with insulin secretion for each condition. C and D, fatty acid oxidation in 832/13 cells transfected with siControl or siACC1 as measured by ¹⁻¹⁴C oxidation to CO₂ (C) and to acid-soluble metabolites (ASM) (D) at both 2.5 and 12 mM glucose. Results represent mean ± S.E. of three independent experiments; **p* < 0.05 compared with siControl; †*p* < 0.01 compared with siControl + DMM; #*p* < 0.001 compared with siControl; §*p* < 0.001 compared with siACC1.

unchanged at 24 and 48 h after removal of siACC1 transfection media (data not shown). The decreased glucose oxidation and oxygen consumption was not due to changes in mitochondrial content, as citrate synthase activity was unchanged in siACC1-

treated cells (105 ± 5 units/mg protein in siControl compared with 108 ± 2 units/mg protein in siACC1, *p* = 0.52). Finally, immunoblot analysis of cytochrome *c* oxidase IV and voltage-dependent anion channel 1, two common mitochondrial markers, revealed no changes in the levels of these proteins in siACC1-treated versus siControl-treated cells (data not shown).

Because ACC1 appears to play an important role in regulating glucose oxidation, it was hypothesized that siACC1 would alter the levels of tricarboxylic acid cycle intermediates/organic acids and short-chain acylcarnitines, which are products of mitochondrial fuel metabolism. Fig. 3, B and C, demonstrates that intracellular levels of all of the organic acids measured by GC/MS increased in control cells in response to stimulation with 12 mM glucose, as we had also reported previously (15, 29). However, in siACC1-treated cells incubated in 12 mM glucose, pyruvate (Fig. 3B), α-ketoglutarate, and malate (Fig. 3C) were all significantly decreased. Consistent with the decrease in glucose oxidation, the short-chain acylcarnitines C2 (acetyl), C4 (butyryl), and C4-OH (β-hydroxybutyryl) measured by MS/MS were all significantly decreased at 12 mM glucose in siACC1-treated compared with siControl-treated cells (Fig. 3D). C2 acylcarnitine is considered to be a surrogate marker for mitochondrial acetyl-CoA levels (15, 29), whereas C4 and C4-OH acylcarnitines are in equilibrium with butyryl and β-OH-butyl-CoAs, respectively. In sum, all of the MS-based metabolite assays summarized in Fig. 3 are consistent with suppression of glucose oxidation in siACC1-treated cells.

ACC1 siRNA Impairs Pyruvate Cycling and the Glucose-induced ATP:ADP Ratio Increase—We have demonstrated previously a strong

correlation between pyruvate cycling and GSIS (9, 15, 18, 29) and have reported that the membrane-permeant malate ester, dimethylmalate (DMM), enhances insulin secretion and pyruvate cycling (9, 18). To test the potential role of pyruvate

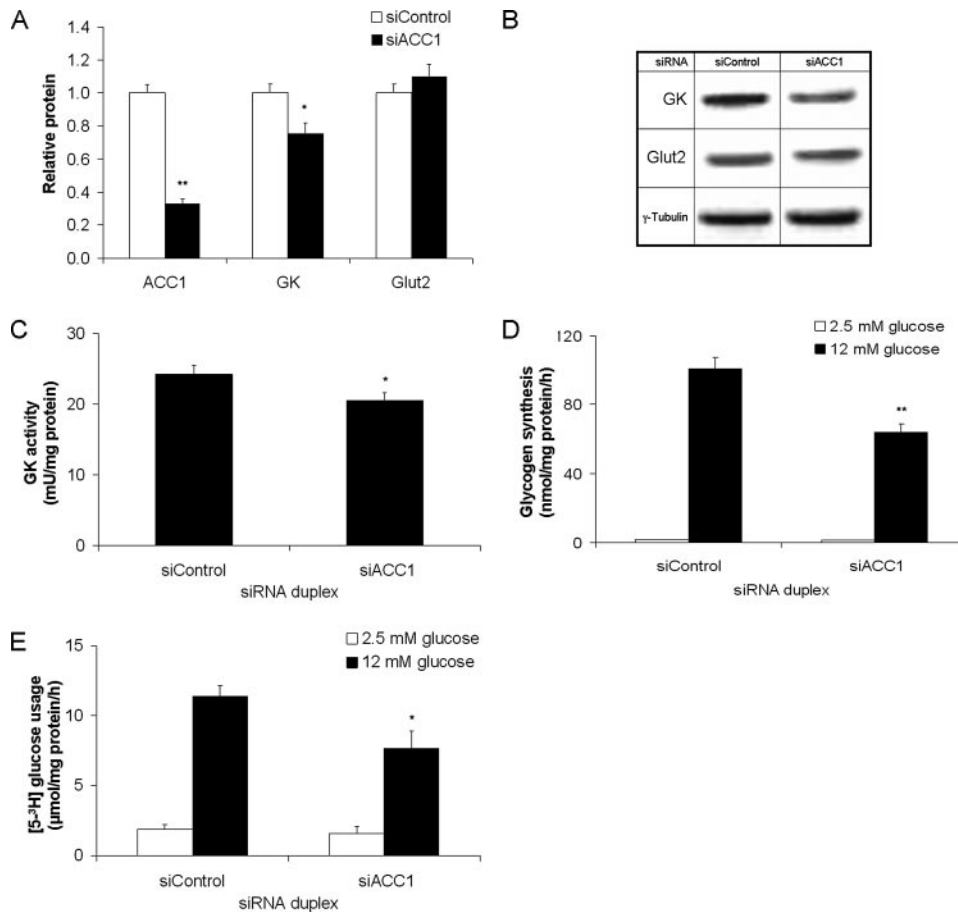


FIGURE 5. Suppression of ACC1 expression decreases GK expression, glycogen synthesis, and glucose flux. A, Glut2 and GK protein levels were measured via immunoblot analysis using γ -tubulin as a loading control. Results represent mean \pm S.E. of eight independent experiments. B, a representative blot is shown. C, GK activity was measured in siACC1 and siControl-treated cells as described under "Experimental Procedures." D, following incubation in [U- 14 C]glucose, glycogen synthesis was measured and normalized to protein content and time. E, following incubation in [5- 3 H]glucose, glycolytic flux was measured and normalized to protein content and time. Results represent mean \pm S.E. of three independent experiments; *, $p < 0.05$; **, $p < 0.001$.

cycling in mediating the effects of ACC1 suppression, siACC1- or siControl-treated 832/13 cells were incubated in 2.5 or 12 mM [U- 13 C]glucose, and pyruvate cycling was analyzed via 13 C NMR-based isotopomer analysis. As shown in Fig. 4A, the increment in pyruvate cycling induced by a switch from 2.5 to 12 mM glucose was decreased by $47 \pm 15\%$ in siACC1-treated cells compared with siControl-treated cells, demonstrating a clear effect of ACC1 suppression on pyruvate cycling activity.

In siControl-treated 832/13 cells, increasing the glucose concentration from 2.5 to 12 mM caused a 70% increase in ATP:ADP ratio concurrent with a 4.8 ± 0.6 -fold increase in insulin secretion (Fig. 4B). Incubation of siControl-treated cells with DMM enhanced insulin secretion at stimulatory glucose, as we have reported previously (9), with a small but not significant increase in the ATP:ADP ratio. Treatment of 832/13 cells with siACC1 caused a clear suppression of GSIS and a concurrent decrease in the ATP:ADP ratio. Remarkably, the addition of DMM to siACC1-treated cells had no restorative effect on ATP:ADP ratio at either glucose concentration. However, it increased the insulin secretion fold-response to glucose in siACC1-treated cells from 2.8 ± 0.4 to 4.2 ± 0.7 , indicating that

partial restoration of GSIS in siACC1-treated cells was independent of ATP:ADP, which could probably be explained by enhanced pyruvate cycling.

Suppression of ACC1 Expression Has No Effect on Fatty Acid Oxidation—ACC1 produces malonyl-CoA, but the ACC1-derived pool of this metabolite is thought to be utilized primarily for lipogenesis, whereas ACC2-derived malonyl-CoA is ascribed the more prominent role in allosteric regulation of CPT1 (2, 3). Consistent with this idea, glucose clearly inhibited palmitate oxidation to CO_2 (Fig. 4C) and acid-soluble metabolites (Fig. 4D, ASM) in both siACC1- and siControl-treated cells. However, siACC1 treatment had no effect on fatty acid oxidation to either CO_2 or acid-soluble metabolites in 832/13 cells at either glucose concentration.

ACC1 siRNA Decreases Glucokinase Protein—To gain insight into the mechanism by which suppression of ACC1 expression affects insulin secretion, several genes with previously described roles in GSIS were measured by reverse transcription-PCR. Treatment of 832/13 cells with siACC1 decreased ACC1 mRNA levels by $52 \pm 7\%$ and caused a $31 \pm 8\%$ decrease in GK mRNA

levels. In contrast, siACC1 treatment caused no significant changes in hexokinase 1, lactate dehydrogenase β , the homeobox transcription factor Nkx6.1, or pancreatic and duodenal homeobox factor 1 (Pdx-1) mRNA levels (data not shown). Immunoblot analysis of 832/13 cells demonstrated that siACC1 treatment decreased GK protein by $25 \pm 7\%$ (Fig. 5A, representative blot Fig. 5B) and GK enzymatic activity by $16 \pm 6\%$ (Fig. 5C) but did not affect Glut2 protein levels. Consistent with the decrease in GK activity in siACC1-treated cells, at 12 mM glucose, glycogen synthesis was decreased by $27 \pm 6\%$ (Fig. 5D), and [5- 3 H]glucose usage was decreased by $33 \pm 12\%$ (Fig. 5E). Taken together, these data indicate that chronic ACC1 suppression may inhibit glucose metabolism by suppressing GK.

Chronic but Not Acute Pharmacologic Inhibition of ACC1 Decreases GSIS—The non-isoform selective ACC inhibitor TOFA has previously been reported to inhibit insulin secretion in parental INS-1 cells (39), but the mechanism of this effect has not been elucidated. To determine whether TOFA exposure would cause similar effects as molecular suppression of ACC1 expression, 832/13 cells were cultured in 0.1% DMSO or 10 μM TOFA for 72 h followed by measurement of [U- 14 C]glucose

Chronic Suppression of ACC1 Impairs Insulin Secretion

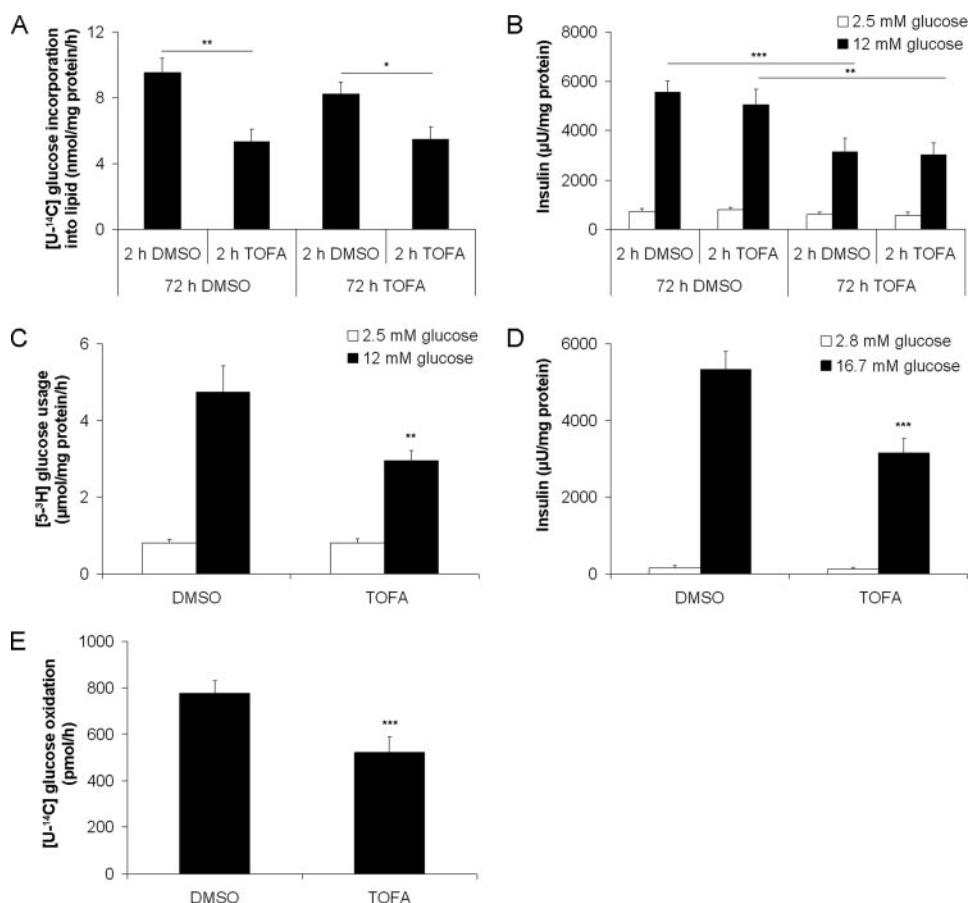


FIGURE 6. Chronic, but not acute, inhibition of ACC1 with TOFA impairs insulin secretion and glucose metabolism in 832/13 cells and rat islets. 832/13 cells were cultured in 0.1% DMSO ± 10 μM TOFA for 72 h, washed, and then cultured in [U-¹⁴C]glucose in the presence or absence of TOFA for an additional 2 h. *A*, glucose incorporation into lipids; *B*, glucose-stimulated insulin secretion; and *C*, [5-³H]glucose usage were measured at 2.5 and 12 mM glucose. *D*, rat islets were cultured in 1% DMSO ± 100 μM TOFA for 72 h, and GSIS was performed at 2.8 and 16.7 mM glucose for 2 h in the absence of TOFA. *E*, rat islets cultured in DMSO or TOFA for 72 h were dispersed, and [U-¹⁴C]glucose oxidation was measured at 16.7 mM glucose. Results represent mean ± S.E. of three independent experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

incorporation into lipids in the continued presence or absence of 10 μM TOFA for 2 h. As shown in Fig. 6A, addition of TOFA only during the 2-h glucose incorporation assay caused a ~50% decrease in glucose incorporation into lipids in both DMSO- and TOFA-pretreated cells. Conversely, acute removal of TOFA for 2 h after 72 h of preincubation with the drug in culture medium resulted in full restoration of normal rates of glucose incorporation into lipids. These data demonstrate that TOFA is an acute and reversible inhibitor of ACC in 832/13 cells. Insulin secretion was also measured in the 832/13 cells exposed to TOFA for varying time periods. As shown in Fig. 6B, acute (2 h) addition of TOFA to the insulin secretion media had no significant effect on GSIS, despite its acute effect to impair lipogenesis. However, in cells in which TOFA was included in culture media for 72 h but then removed for the subsequent 2-h insulin secretion assay, insulin secretion was decreased by 43 ± 11% at 12 mM glucose. Cells that were treated with TOFA for both the 72-h preincubation period and for the 2-h insulin secretion assay exhibited a similar 46 ± 10% decrease in insulin secretion at 12 mM glucose.

These data demonstrate that the block of lipogenesis caused by acute (2 h) exposure of 832/13 cells to TOFA is not sufficient

to cause impairment of GSIS, and conversely, that restoration of lipogenesis by acute removal of the drug after chronic exposure is not sufficient to rescue glucose sensing. Taken together, these data support the idea that inhibition of ACC impairs GSIS via suppression of glucose metabolism rather than via its effects on new lipid synthesis. Consistent with this model, TOFA treatment of 832/13 cells for 72 h caused a 38 ± 13% decrease in [5-³H]glucose usage (Fig. 6C) and a 27 ± 10% decrease in GK protein levels ($p < 0.05$; data not shown), similar to what we reported earlier in siACC1-treated cells. Also, treatment of isolated rat islets with TOFA for 72 h caused a 41 ± 9% decrease in GSIS, consistent with our findings in the 832/13 cell line (Fig. 6D). Finally, chronic TOFA treatment reduced glucose oxidation in dispersed islets by 33 ± 8% (Fig. 6E).

DISCUSSION

Acetyl-CoA carboxylase has well known roles in the regulation of lipid metabolism. Mammals express both cytosolic (ACC1) and mitochondria-associated (ACC2) ACC isoforms. Both enzymes catalyze the conversion of acetyl-CoA to malonyl-CoA, which serves both as a substrate for lipogenesis and as a

potent inhibitor of the mitochondrial protein CPT1, which shuttles long-chain fatty acyl-CoAs into the mitochondria for β-oxidation. It has been suggested that malonyl-CoA produced by cytosolic ACC1 is used primarily for *de novo* lipogenesis, whereas by virtue of its mitochondrial localization, the ACC2-derived malonyl-CoA pool serves to regulate CPT1. Studies of isoform-specific ACC knock-out mice have provided evidence for critical regulatory roles of these enzymes in mammalian fuel homeostasis (1–4).

Obesity and type 2 diabetes, which have reached epidemic proportions in many countries of the world, are associated with ectopic lipid accumulation in non-adipose tissues and organs such as islets, liver, and muscle. Modern obesity is caused by ingestion of excess quantities of both glucose and fat coupled with a sedentary life style. High rates of carbohydrate intake from sugar-laden soft drinks and other sources provide glucose, which can increase mitochondrial citrate production, leading to increased flux through the two ACC enzymes. This can lead in turn to simultaneous stimulation of lipogenesis and inhibition of fatty acid oxidation. This coupled with the interesting features of ACC knock-out mice (1–4) has led to a strong interest in developing pharmacologic inhibitors of the ACC

enzymes as a means of combating excess lipid accumulation in obesity and type 2 diabetes.

One concern about the development of ACC inhibitors for obesity and diabetes treatments is that their potential impact on metabolic regulation of insulin secretion in the pancreatic β -cell is incompletely understood. Previous studies have shown that stable expression of an ACC1-specific antisense construct impairs GSIS in INS-1 cells (5). Similarly, treatment of INS-1 cells with TOFA, which inhibits both ACC isoforms at micromolar concentrations, also is reported to impair GSIS in INS-1 cells (39). However, in both of these cases it was assumed that the inhibitory effect of ACC suppression on GSIS was because of blockade of *de novo* lipogenesis and/or activation of fatty acid oxidation in response to reductions in malonyl-CoA levels. Such an interpretation was guided by earlier studies suggesting that malonyl-CoA serves a stimulus/secretion coupling factor for GSIS, possibly via its capacity to suppress β -cell fatty acid oxidation and expand the cytosolic fatty acyl-CoA pool (11, 12). However, several recent studies are not consistent with this model. Thus, blockade of the normal glucose-induced rise in malonyl-CoA by overexpression of malonyl-CoA decarboxylase in 832/13 cells, INS-1 cells, or primary rat islets has no effect on GSIS (22–24). Moreover, neither siRNA-mediated knockdown of fatty acid synthase in 832/13 cells or rat islets (40) nor knock-out of fatty acid synthase in transgenic mice (41) affects GSIS. Also, siRNA-mediated knockdown of ATP-citrate lyase, which produces cytosolic acetyl-CoA, has no effect on GSIS in 832/13 cells or primary rat islets, despite potent suppression of [U - ^{14}C]glucose incorporation into lipids (40). Other groups have reported a small decrease in GSIS at intermediate glucose concentrations in 832/13 cells treated with siRNA specific to citrate lyase, but these authors did not validate their findings in primary rat islets (42). Here we show that the effects of ACC1 suppression on GSIS require chronic suppression of the enzyme. In contrast, acute inhibition of ACC1 via addition of TOFA had no impact on GSIS, despite dramatic effects of the drug on ACC activity as measured by glucose incorporation into lipids. These findings align with the growing body of evidence suggesting that neither malonyl-CoA synthesis nor *de novo* lipogenesis is an acute regulator of GSIS.

The current study demonstrates an alternative mechanism for ACC1-mediated inhibition of GSIS involving the impairment of glucose metabolism. Chronic inhibition of ACC1 led to decreased GK protein levels, which occurred in concert with reduced rates of glucose usage, decreased glucose oxidation, and decreased levels of tricarboxylic acid cycle intermediates and short-chain acylcarnitines. The fall in C2 (acetyl) carnitine levels indicates that acetyl-CoA was lower in siACC1-treated cells, possibly contributing to the decrease in pyruvate cycling activity by lowering of a key allosteric activator of PC (29). The mechanism by which ACC1 suppression leads to decreases in GK protein and glucose flux was not elucidated in these studies and remains to be studied. The changes in glucose metabolism also led to decreases in the ATP:ADP ratio and pyruvate cycling activity. Interestingly, the addition of a pyruvate cycling metabolite, DMM, rescued GSIS without restoring the normal ATP:ADP ratio. This is now a third example from among our studies of a “disconnect” between the manipulation of GSIS and ATP

and ADP levels. In one prior study, we showed that knockdown of PC had no effect on GSIS despite a 30% suppression of ATP levels at high glucose (29). In a second study, suppression of the citrate/isocitrate carrier caused strong inhibition of GSIS with no effects on ATP or ADP levels (43). Although surprising in the context of the long held view of a critical role for changes in ATP:ADP ratio and regulation of the K_{ATP} channel system in GSIS (44), our recent findings are consistent with studies showing that transgenic mice lacking expression of the sulfonylurea receptor of the K_{ATP} channel complex exhibit only modest perturbations in GSIS (45, 46). These newer findings could suggest: 1) compensatory activity of another ion channel; 2) a more prominent role for K_{ATP} channel-independent signaling pathways and pyruvate cycling in control of GSIS than commonly appreciated; 3) utilization of other kinds of stimulus/secretion coupling factors such as reactive oxygen species (47).

The most widely used chemical ACC inhibitors are nonselective. Delivery of these compounds to C2C12 cells and Sprague-Dawley rats results in increased fatty acid oxidation in excised muscle samples (48) and improved muscle insulin sensitivity (49); however, there are data suggesting that their use may result in diminished β -cell function (50). The current study supports these latter observations and provides a cautionary note for the use of ACC inhibitors for obesity and diabetes therapy, as their prolonged administration may lead to a form of β -cell dysfunction and impaired GSIS caused by interference in β -cell glucose metabolism.

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