Compensatory Responses to Pyruvate Carboxylase Suppression in Islet β-Cells

PRESERVATION OF GLUCOSE-STIMULATED INSULIN SECRETION*

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Mette V. Jensen‡, Jamie W. Joseph‡, Olga Ilkayeva‡, Shawn Burgess‡, Danhong Lu‡, Sarah M. Ronnebaum‡, Matthew Odegaard‡, 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 and ¶Department of Medicine, Duke University Medical Center, Durham, North Carolina 27704 and ¶Advanced Imaging Research Center and the Department of Radiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235

We have previously reported that glucose-stimulated insulin secretion (GSIS) is tightly correlated with pyruvate carboxylase (PC)-catalyzed anaplerotic flux into the tricarboxylic acid cycle and stimulation of pyruvate cycling activity. To further evaluate the role of PC in β-cell function, we constructed a recombinant adenovirus containing a small interfering RNA (siRNA) specific to PC (Ad-siPC). Ad-siPC reduced PC mRNA levels by 83 and 64% and PC protein by 56 and 35% in INS-1-derived 832/13 cells and primary rat islets, respectively. Surprisingly, this manipulation did not impair GSIS in rat islets. In Ad-siPC-treated 832/13 cells, GSIS was slightly increased, whereas glycolytic rate and glucose oxidation were unaffected. Flux through PC at high glucose was decreased by only 20%, suggesting an increase in PC-specific activity. Acetyl carnitine, a surrogate for acetyl-CoA, an allosteric activator of PC, was increased by 36% in Ad-siPC-treated cells, suggesting a mechanism by which PC enzymatic activity is maintained with suppressed PC protein levels. In addition, the NADPH:NADP ratio, a proposed coupling factor for GSIS, was unaffected in Ad-siPC-treated cells. We conclude that β-cells activate compensatory mechanisms in response to suppression of PC expression that prevent impairment of anaplerosis, pyruvate cycling, NADPH production, and GSIS.

Glycolytic and mitochondrial metabolism of glucose is essential for generating the cellular signals that lead to glucose-stimulated insulin secretion (GSIS)2 from pancreatic islet β-cells. The most well characterized coupling factor for GSIS is the cytosolic ATP:ADP ratio. It is established that this ratio increases in response to increased glucose metabolism in β-cells, resulting in closure of ATP-sensitive K+ (KATP) channels, plasma membrane depolarization, and activation of voltage-dependent Ca2+ channels. The resulting increase in cellular calcium promotes exocytosis of insulin-containing granules (reviewed in Refs. 1 and 2). However, significant GSIS also occurs under conditions where closure of KATP channels is prevented, demonstrating an important role for KATP channel-independent pathways for fuel-regulated insulin granule exocytosis (3).

Pancreatic β-cells have a remarkable capacity for anaplerotic influx of substrates into the tricarboxylic acid cycle due to high levels of the biotin-dependent enzyme pyruvate carboxylase (PC), which converts pyruvate to oxaloacetate (OXA). It has been estimated that ~40–50% of pyruvate enters β-cell mitochondrial metabolism through PC at stimulatory glucose concentrations (4–7). PC-mediated anaplerosis plays a central role in β-cell function, as supported by the following observations. 1) PC-mediated pyruvate exchange with tricarboxylic acid cycle intermediates (“pyruvate cycling”), rather than the fractional contribution of glucose to acetyl-CoA formation (pyruvate dehydrogenase-catalyzed pyruvate metabolism), correlates with insulin secretion in a set of INS-1-derived cell lines with varying capacities for GSIS (5). 2) In lipid-cultured 832/13 insulinoma cells, the normal glucose-induced increment in pyruvate cycling is abolished in concert with profound suppression of GSIS (8). 3) Phenylacetic acid, a competitive inhibitor of PC, decreases pyruvate cycling in INS-1-derived cell lines and also inhibits GSIS from both β-cell lines and islets (5, 9, 10). 4) Culture of MIN6 cells or islets in the presence of a specific agonist for liver X receptor for 72 h induces expression of several genes, including PC. This treatment also results in increased GSIS, an effect that is abolished in the presence of phenylacetic acid (11).

A potential role of PC-mediated pyruvate cycling in β-cells may be to generate NADPH, a suggested coupling factor for GSIS (12, 13). In support of this idea, we have recently demonstrated that siRNA-mediated silencing of the mitochondrial citrate/isocitrate carrier resulted in a dramatic decrease in GSIS and a corresponding reduction of the NADPH:NADP ratio in β-cells (14). Similarly, siRNA-mediated suppression of cytosolic, NADP-dependent isocitrate dehydrogenase caused impairment of GSIS in concert with a decrease in pyruvate cycling flux and NADPH:NADP ratio (15). In the present study, we have continued our investigation of the role of PC-mediated anaple-
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The suppression of pyruvate carboxylase (PC) expression is known to impact glycolysis and pyruvate cycling in control of GSIS by applying siRNA technologies to manipulate the expression levels of PC in INS-1-derived 832/13 cells and primary rat islets. Surprisingly, siRNA-mediated suppression of PC protein expression did not impair GSIS in either cell lines or primary cells. This lack of impact on GSIS appears to be explained by an increase in acetyl-CoA levels, which in turn allosterically activates PC, thereby largely maintaining flux through the enzyme despite lower expression. We also present evidence consistent with the idea that flux through the cytosolic, NADP-dependent isocitrate dehydrogenase is maintained at the expense of flux through the mitochondrial, NAD-dependent isocitrate dehydrogenase enzyme, thus maintaining NADPH levels and preventing GSIS from declining. These studies identify compensatory mechanisms by which the β-cell defends against suppression of anaplerotic flux, pyruvate cycling, cytosolic NADPH production, and ultimately GSIS in response to lowering of PC expression.

**EXPERIMENTAL PROCEDURES**

**Reagent**—All reagents were obtained from Sigma unless otherwise indicated.

**Pancreatic β-Cell Lines**—We have previously described the isolation of subclones of the rat insulinoma INS-1 cell line (16) via a transfection/selection strategy (17). Cell lines 832/13 and 832/3, which exhibit robust GSIS, were used in the current study and were cultured as previously described (17, 18).

**siRNA Duplex-mediated Gene Silencing**—Expression of rat PC (Gen Bank™ sequence accession number NM_012744) was suppressed by introducing siRNA duplexes (Applied Biosystems, Foster City, CA) at a final concentration of 0.5 μg/10⁶ cells into 832/13 and 832/3 insulinoma cells using the Amaxa nucleofection system (Amaxa Inc., Gaithersburg, MD). The PC-targeting sequences used were GGT GCC CAA GGA GAA TGG T (siPC1), GGA GAA TGG TGT AGA TGCT (siPC2), and GGA AAA CAT CCG AAT CAA T (siPC3). A duplex with no known gene homology was used as control (siControl; GAG GGA AAA CAT CCG AAT CAA T). After nucleofection, cells were treated at 20–25% confluency with Ad-siPC at one of two doses (10 × 10⁶ plaque-forming units/ml of medium, Ad-siPC, low; or 20 × 10⁶ plaque-forming units/ml, Ad-siPC, high) or Ad-siControl at a dose of 20 × 10⁶ plaque-forming units/ml. After 16 h of exposure, virus-containing medium was removed, and cells were cultured for an additional 72 h before analysis.

**Quantification of PC mRNA Levels Using Real-time PCR**—RNA was isolated using the Qiagen RNeasy kit (Qiagen Inc., Valencia, CA) and reverse transcribed with iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s recommendations. Quantitative real-time PCR analysis of PC mRNA levels was performed using the TaqMan® gene expression assay from Applied Biosystems. As a loading control, prevalidated primers and probes against rat cyclophilin B were included (Applied Biosystems). Measurements were performed with the ABI Prism 7000 sequence detection system.

**Quantification of PC Protein Levels Using Streptavidin Blotting**—Adenovirus-treated cells or rat islets were lysed in 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% IGEval CA-630, and cleared protein lysates were loaded onto denaturing NuPAGE 3–8% Tris-acetate gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Invitrogen). After blocking overnight in 5% milk diluted in Tris-buffered saline/0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated streptavidin (Chemicon, Temecula, CA) diluted 1:4000 in Tris-buffered saline/0.1% Tween 20 containing 5% bovine serum albumin (BSA) for 45 min. Membranes were washed for 4 × 5 min in Tris-buffered saline/0.1% Tween 20, and PC levels were quantified on a VersaDoc™ Model 4000 imaging system, using the biotin-binding protein propionyl-CoA carboxylase as a loading control (Bio-Rad).

**Insulin Secretion and Content in INS-1-derived Cells**—Cells treated with duplexes or viruses as described above and grown to confluency were washed with PBS and preincubated for 1.5 h in secretion buffer (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES, 2.5 mM CaCl₂, 0.2% BSA, pH 7.2) containing 2.5 mM glucose. For GSIS, cells were incubated in secretion buffer for an additional 2 h in the presence of 2.5 or 12 mM glucose as indicated, followed by collection of buffer samples for insulin radioimmunoassay. For insulin content measurements, cells were lysed in 0.1 M acetic acid, 0.1% BSA under mild sonication on ice. After centrifugation for 5 min at 12,000 × g at 4 °C, the extracts were neutralized by dilution in PBS. Insulin levels in secretion buffer and extracts were quantified by insulin radioimmunoassay with the Coat-A-Count kit (DPC, Los Angeles, CA).

**Insulin Secretion during Perfusion of 832/13 Cells**—Cells seeded onto 25-mm glass coverslips placed in 6-well plates were treated with various adenoviruses as described above. Prior to perfusion, 832/13 cells were incubated for 1.5 h in secretion buffer containing 2.5 mM glucose at 37 °C. Two glass coverslips were then loaded into a closed cell perfusion chamber (Warner Instruments, Hamden, CT), and cells were perfused at a flow rate of 1 ml/min using a Minipuls 3 pump (Gilson, Middleton, WI). The temperature was maintained at 37 °C using an in-line solution heater (Warner Instruments). Cells were pre-perfused for 30 min with secretion buffer containing 2.5 mM glucose prior to the sample collection. For studies of secretion in the presence of high K⁺, the following buffer was used: 84 mM NaCl, 35 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES, 2.5 mM CaCl₂, 0.2% BSA, pH 7.2.

**Insulin Secretion from Rat Islets**—Islets were harvested from male Sprague-Dawley rats weighing ~250 g as previously described (20, 21). Once harvested, islets were treated for 20 h with Ad-siPC or Ad-siControl adenoviruses at a dose of 10⁵ plaque-forming units/islet. After removal of virus-containing medium, islets were cultured for an additional 72 h with medium changes every 24 h. GSIS (1 h of incubation at 2.5 mM glucose) was measured using a radioimmunoassay. An additional 24 h of incubation in the presence of medium (no glucose) was done to normalize for basal insulin release. Secretion media were collected at the end of each 1 h incubation period.
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glucose followed by 1 h of incubation at 16.7 mM glucose) was measured with 35 islets for each condition. Subsequently, islets were collected, washed with PBS, and lysed for analysis of PC expression by real-time PCR analysis (10 islets/sample) or streptavidin-based protein quantification (30 islets/sample) as described above.

Glucose Usage and Glucose Oxidation Measurements—832/13 cells were treated with recombinant adenoviruses, cultured in 12-well plates, and pretreated identically as described for GSIS analysis with the exception that either [5-3H]glucose (specific activity of 0.06 Ci/mol) or [U-14C]glucose (specific activity of 0.2 Ci/mol) was included as tracer for glucose usage and glucose oxidation measurements, respectively. The analyses were performed as previously described (22, 23).

Metabolic Profiling of Intracellular Organic Acids and Aciylcarnitines—For quantitative gas chromatography/mass spectrometry (GC/MS)-based analysis of intracellular organic acid levels, virus-treated cells were cultured in 15-cm dishes and incubated for 1.5 h in secretion buffer containing 2.5 mM glucose followed by 2 h of incubation at either 2.5 or 12 mM glucose. Subsequently, cells were lysed in ice-cold 0.1 M HCl containing internal standards (3H2-lactate; C/D/N Isotopes, Pointe-Claire, Quebec), 13C3-pyruvate (Aldrich), 3H4-succinate (Aldrich), 3H2-fumarate (C/D/N Isotopes), 13C1-malate (IsoTec; St. Louis, MO), 3H6-α-ketoglutarate (IsoTec), and 3H4-citrate (IsoTec). Cell remnants were scraped and collected, and pH of the cleared cell extract was adjusted to 8–9 with KOH. Ethoxyamine (Acros Organic, Belgium) was added, and the mixture was incubated at room temperature for 30 min to complete ethoxyamination of ketoacids. Next, pH was adjusted to 1–2 with HCl, 50 mg of NaCl was added, and samples were vortexed and extracted twice with ethyl acetate (VWR, West Chester, PA). Upper phases containing the organic acids were combined and subsequently dried under nitrogen. 4 μl of pyridine and 50 μl of N,O-Bis(trimethylsilyl)trifluoroacetamide were added, and the samples were incubated at 90 °C for 30 min. The trimethylsilyl derivatives were separated by gas chromatography on a Thermo Finnigan Trace GC Ultra and quantified by selected ion monitoring on a Thermo Finnigan Trace DSQ using stable isotope dilution. For measurement of acylcarnitine levels, 832/13 cells were seeded in 12-well plates, treated with recombinant adenoviruses, and cultured for 72 h. 24 h before harvesting, l-carnitine (inner salt) was added to the growth medium to a final concentration of 1 mM. Cells were cultured in 15-cm dishes were preincubated in the presence of 2.5 mM glucose for 1 h, followed by incubation in secretion buffer containing 2.5 or 12 mM [U-13C]glucose (Cambridge Isotope Laboratories, Cambridge, MA) for 4 h. Cells were washed once with cold PBS and extracted with cold 3.5% perchloric acid. Extracts from three dishes/condition were pooled, neutralized, and lyophilized prior to 13C NMR analysis. The principle in the method is that by analyzing the 13C-isotopomer pattern of glu-tamate a direct measure of pyruvate cycling (PC-mediated pyruvate exchange with tricarboxylic acid cycle intermediates) relative to tricarboxylic acid cycle flux can be obtained (5). In short, lyophilized samples were dissolved in 2H2O, and proton-decoupled 13C NMR spectra were recorded on a 600-MHz 14T Varian INOVA NMR spectrometer using a 45° pulse and a 3-s repetition time in a 5-mm tunable broadband probe. The areas of the multiplets arising from 13C to 13C spin-spin coupling in the glutamate C2, C3, and C4 resonances were determined using the line-fitting routine in the NMR program NUTS (Acorn NMR, Fremont, CA), and 13C-isotopomer analysis was performed with tcaCALC (5).

NADPH and NADP Analyses—Levels of nicotinamide adene-dine dinucleotide phosphates were measured in Ad-siControl and Ad-siPC-treated cells using an enzymatic cycling method adapted from Passonneau and Lowry (25). Following GSIS, virus-treated cells cultured in 6-well plates were scraped, centrifuged for 10 s at 10,000 × g, 4 °C, and cell pellets were frozen in a dry ice/ethanol bath. Cells were thawed on ice, resuspended in 40 mM NaOH/5 mM cysteine, sonicated for 10 s, and centrifuged for 1 min at 10,000 × g at 4 °C. Each sample was divided into three, and 0.3 mM HCl (NADPH is destroyed by low pH), 40 mM NaOH/5 mM cysteine (NADP is destroyed at high pH), and H2O were added for NADP, NADPH, and total NADP(H) determinations, respectively. Samples for NADPH and NADP determination were incubated at 60 °C for 15 min, whereas samples for measuring total NADP(H) levels were kept on ice during the incubation time. NADPH standards (0.25–4 μM) were treated in parallel. Subsequently, cycling reagent (50 mM imidazole, 50 mM imidazole-HCl, 5 mM glucose 6-phosphate, 7.5 mM disodium α-ketoglutarate, 0.1 mM ADP, 25 mM ammonium acetate, 0.1% BSA, 1.5 units/ml of Leucanostoc glucose-6-phosphate dehydrogenase, 1.5 units/ml of beef liver glutamic dehydrogenase) was added, and after 3 h of incubation at 37 °C, samples and standards were boiled for 3 min and centrifuged at 10,000 × g for 10 min. The amount of 6-phosphogluconolactone was determined by adding indicator reaction buffer (50 mM imidazole, 50 mM imidazole-HCl, 30 mM ammonium acetate, 2 mM MgCl2, 0.1 mM EDTA, 300 μM NADP, 0.5 units/ml of Torula yeast gluconate dehydrogenase) to the cycling reactions, and absorbance at 340 nm was read after the kinetic reaction was completed.

ATP Determinations—832/13 cells cultured in 6-well plates and treated with either Ad-siControl or Ad-siPC were washed with PBS, scraped, and pelleted by centrifugation at 8,000 × g, 4 °C for 10 s. After a quick freeze in a dry ice/ethanol bath, cell pellets were resuspended by adding 10% (w/v) perchloric acid to the thawed cell pellets. After centrifugation, the supernatants were neutralized with 2 N KOH/0.5 M triethanolamine. Aliquots of each deproteinized sample were assayed for ATP with Luciferase kit (Sigma) according to the manufacturer’s instructions.

Statistics—Data were expressed as the mean ± S.E. of at least three independent experiments performed in triplicate. Statistical significance was determined using a two-tailed Student’s t test, assuming equal variances. p < 0.05 was considered as significant.
RESULTS

Suppression of PC Expression with siRNA Duplexes—We have reported previously that GSIS is tightly correlated with PC-catalyzed anaplerotic influx into the tricarboxylic acid cycle and further participation of OXA in various pyruvate cycling pathways (5, 8, 15). To directly test the impact of PC-catalyzed anaplerosis on GSIS, we designed multiple siRNA duplexes specific for the rat PC mRNA sequence. When introduced into the INS-1-derived cell line 832/13 by nucleofection, one of the siRNA duplexes had no significant effect on PC mRNA levels (siPC1), whereas two other duplexes (siPC2 and siPC3) suppressed PC mRNA levels by 88 ± 1% and 86 ± 1%, respectively (Fig. 1). Combined treatment with duplexes siPC2 and siPC3 had no additional effect on PC expression. Surprisingly, none of the duplexes had any effect on GSIS compared with cells transfected with a nonspecific siRNA sequence (siControl). Similar results were obtained in an independent, robustly glucose-responsive cell line, 832/3 (data not shown).

To further investigate the role of PC in GSIS, we constructed recombinant adenoviruses containing the siPC2 (Ad-siPC) or the siControl (Ad-siControl) sequence. 832/13 cells were treated with two different doses of Ad-siPC resulting in 73 ± 3% (Ad-siPC, low) and 83 ± 1% (Ad-siPC, high) decreases in PC mRNA levels, respectively, compared with cells treated with Ad-siControl (Fig. 2A; p < 0.001). To estimate the corresponding reduction in PC protein, we performed streptavidin-based immunoblot analysis, taking advantage of the biotin binding ability of PC (26) (Fig. 2B). This analysis demonstrated decreases in PC protein levels of 40 ± 4% (Ad-siPC, low) and 56 ± 2% (Ad-siPC, high), respectively, compared with Ad-siControl (p < 0.001; Fig. 2C).

Effect of PC Silencing on GSIS from 832/13 β-Cells and Rat Islets—Surprisingly, Ad-siPC administration to 832/13 cells and suppression of PC protein to the levels shown in Fig. 2 caused a modest but significant increase in GSIS (Fig. 3A). This effect was largely due to an increase in insulin secretion at stimulatory glucose (from 5459 ± 361 microunits/mg of protein in Ad-siControl-treated cells to 6971 ± 449 microunits/mg of protein and 6943 ± 450 microunits/mg of protein in Ad-siPC, low- and Ad-siPC, high-treated cells, respectively (p < 0.001).

The enhancement of insulin secretion at stimulatory glucose was not explained by changes in insulin content (35 ± 2 microunits/mg of protein for Ad-siControl versus 35 ± 2 microunits/mg of protein and 39 ± 3 microunits/mg of protein for Ad-siPC, low (p = 0.98) and Ad-siPC, high (p = 0.25), respectively).

We next investigated whether PC manipulation affected the dynamics of insulin secretion in cell perfusion studies. Given the very similar effects of the high and low doses of Ad-siPC on GSIS in the static incubation experiments shown in Fig. 3A,
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these and all subsequent experiments were performed only with the high viral dose, which caused an average decrease in PC protein of 56 ± 2%. As shown in Fig. 3B, the minute-to-minute insulin output from cells with lower PC expression was similar to the secretion profile for the Ad-siControl cells, both in response to glucose alone and when stimulatory glucose was accompanied by 35 mMK⁺.

Subsequently, we used Ad-siPC to suppress PC expression in rat islets. We obtained a 64 ± 3% knockdown of PC mRNA levels (p < 0.001) with Ad-siPC treatment, translating into a 35 ± 5% reduction in PC protein (p < 0.001) compared with islets treated with Ad-siControl (data not shown). In seven independent experiments, this manipulation had no effect on insulin secretion at either basal (23 ± 4 microunits/35 islet/h for Ad-siPC versus 28 ± 8 microunits/35 islet/h for Ad-siControl, p = 0.4) or stimulatory glucose levels (124 ± 11 microunits/35 islet/h, Ad-siPC versus 121 ± 11 microunits/35 islet/h, Ad-siControl; p = 0.8; Fig. 3C).

Decreased PC Expression Does Not Affect Pyruvate Levels, Glucose Usage, or Glucose Oxidation but Increases Intracellular Lactate Levels—Pyruvate has multiple potential metabolic fates in β-cells, including entry into pyruvate cycling pathways via PC, conversion to lactate, or entry into the tricarboxylic acid cycle for oxidation via pyruvate dehydrogenase. To investigate the impact of Ad-siPC treatment on these various pathways, we measured glucose usage and glucose oxidation as well as the intracellular levels of pyruvate and lactate in 832/13 cells. As expected, Ad-siControl-treated cells exhibited significant increases in glucose usage (Fig. 4A) and oxidation (Fig. 4B) as glucose concentration was raised from 2.5 to 12 mM (p < 0.001). Flux through these pathways was unaffected by Ad-siPC treatment at basal or stimulatory glucose (p > 0.4, three independent experiments). Measurement of intracellular lactate and pyruvate concentrations by quantitative GC/MS revealed large increases in both analytes in Ad-siPC- and Ad-siControl-treated cells in response to increases in glucose levels from 2.5 to 12 mM. However, whereas pyruvate levels (Fig. 4C) were unaffected by PC manipulation at both basal and stimulatory glucose (p > 0.4; three independent experiments), intracellular lactate was increased by 60% in Ad-siPC-treated cells at 12 mM glucose compared with Ad-siControl-treated cells (Fig. 4D; p < 0.01 for three independent experiments).

PC Silencing Has a Minor Impact on Pyruvate Cycling Flux—We next investigated the effect of Ad-siPC treatment on pyruvate cycling activity as measured by 13C NMR. As shown in Fig. 5A and consistent with our prior work (5, 8, 15), an increase in glucose levels from 2.5 to 12 mM caused a significant increase in pyruvate cycling activity in Ad-siControl-treated cells (from 0.45 ± 0.07 to 1.06 ± 0.03; p < 0.001). Ad-siPC treatment caused no significant change in basal pyruvate cycling activity (0.34 ± 0.04; p > 0.2) but resulted in a 20 ± 3% decrease at stimulatory glucose (0.85 ± 0.05; p < 0.005) relative to Ad-siControl-treated cells. However, the increment in pyruvate cycling activity as glucose was raised from basal to stimulatory levels was not significantly different between Ad-siPC- and Ad-siControl-treated cells (Fig. 5B; 0.61 ± 0.07 for Ad-siControl versus 0.51 ± 0.03 for Ad-siPC; p > 0.2, three independent experiments) despite the 56% decrease in PC protein levels in these experiments.

Acetyl Carnitine Levels Are Elevated in 832/13 Cells with Lower PC Expression—Because reduction of PC protein levels did not translate into a corresponding decrease in pyruvate cycling activity, it may suggest an increased specific activity of the remaining PC protein in Ad-siPC-treated cells. It is well recognized that acetyl-CoA esters act as allosteric activators of PC (reviewed in Ref. 27). We reasoned that diversion of pyruvate away from the PC reaction may have resulted in increased flux through pyruvate dehydrogenase and an increase in mito-
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chondrial acetyl-CoA levels. To address this possibility, we applied quantitative MS/MS for measuring cellular levels of acetyl carnitine, which is in close equilibrium with acetyl-CoA. Using this method, 10 carnitine esters (C3, C4, C4OH, C6, C8, C12, C14, C16, C18, and C18:1) in addition to acetyl carnitine (C2) could be detected in 832/13 cells. Among these 11 analytes, only acetyl carnitine was altered in response to Ad-siPC treatment, increasing by 36\%/H11006\% compared with its levels in Ad-siControl-treated cells (p\%/H11021\%< 0.001, three independent experiments) (Fig. 6). This rise in acetyl carnitine, which mirrors acetyl-CoA levels, suggests a mechanism by which PC-specific activity may have increased in cells with siRNA-mediated decreases in PC expression.

NADPH:NADP Ratio Is Maintained in Ad-siPC-treated Cells—NADPH, a byproduct of pyruvate cycling, has been implicated as a coupling factor for GSIS (12, 13, 15). To investigate whether silencing of PC affected NADPH homeostasis, we applied an enzymatic cycling assay for measuring NADPH and NADP levels. As shown in Fig. 7, Ad-siControl-treated cells exhibited a significant increase in NADPH:NADP ratio as glucose was raised from 2.5 to 12 mM (p\%/H11006\%< 0.001). However, despite the metabolic changes described in prior sections, Ad-siPC treatment had no effect on NADPH:NADP ratio or on NADPH concentration (not shown) at basal (p\%/H11013\%> 0.13) or stimulatory glucose (p\%/H11008\%> 0.89; three independent experiments).

Effects of PC Silencing on ATP and Tricarboxylic Acid Cycle Intermediate Levels—Despite the lack of effect of PC suppression on glucose oxidation (Fig. 4B), total ATP levels were decreased by 36\% in Ad-siPC-treated cells (375 ± 65 \mu mol/mg of protein) compared with Ad-siControl (586 ± 64 \mu mol/mg of protein; p\%/H11003\%= 0.03; Fig. 8A). To investigate whether the levels of tricarboxylic acid cycle intermediates were also altered by PC manipulation, we used quantitative GC/MS to measure the concentrations of six analytes (succinate, fumarate, malate, aKG, isocitrate, and citrate, Fig. 8B). Isocitrate could not be detected at basal or stimulatory glucose, and fumarate was undetectable at basal glucose. For the remaining metabolites, a 2–30-fold increase was observed in Ad-siControl-treated cells as glucose was raised from 2.5 to 12 mM. No changes were observed in levels of succinate, fumarate, malate, or citrate in Ad-siPC versus Ad-siControl-treated cells (p\%/H11003\%> 0.3; three independent experiments), whereas aKG was significantly reduced in response to Ad-siPC treatment at both low (6.1 ± 0.8 versus 4.2 ± 0.4 nmol/mg of protein for Ad-siControl; p\%/H11005\%< 0.05) and high (6.1 ± 0.8 versus 4.2 ± 0.4 nmol/mg of protein for Ad-siControl; p\%/H11005\%< 0.05) glucose concentrations.

DISCUSSION

PC catalyzes the ATP-dependent carboxylation of pyruvate to form OXA. The enzyme is localized in the mitochondrial matrix, and only one PC isoform has been reported in mammals, consisting of four identical subunits of ~130 kDa (28–30). PC is highly expressed in liver and kidney, where it works with phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase to facilitate gluconeogenesis. PC is also abundant in adipose tissue and lactat-
ing mammary gland, where it plays a key role in de novo fatty acid biosynthesis via regeneration of glycerol phosphate through phosphoenolpyruvate carboxykinase and by exporting mitochondrial acetyl groups into the cytosol as citrate for generation of NADPH (31, 32).

Islet β-cells also contain high levels of PC but lack phosphoenolpyruvate carboxykinase and exhibit relatively low lipogenic capacity (33, 34). An important clue to the role of PC in β-cell metabolism came with the discovery that these cells express enzymes that allow “cycling” of pyruvate via PC-catalyzed carboxylation. Thus, OXA generated via PC is converted to malate, citrate, or isocitrate in the tricarboxylic acid cycle and subsequently reconverted to pyruvate via several possible combinations of cytosolic and mitochondrial enzymes (Fig. 9) (5, 13, 15, 35). Furthermore, application of 13C NMR-based mass isotopomer techniques has revealed that pyruvate cycling flux correlates with the capacity for GSIS in a set of variously glucose-responsive INS-1-derived cell lines (5). Changes in PC-mediated pyruvate cycling have also been implicated in lipid-induced impairment of GSIS. Long-term exposure of mouse

FIGURE 5. Effects of PC silencing on pyruvate cycling flux in 832/13 cells. Pyruvate cycling flux, measured by 13C NMR isotopomer analysis, was compared in Ad-siControl- and Ad-siPC-treated cells. A, changes in pyruvate cycling activity in response to glucose. B, increment in pyruvate cycling as glucose is raised from 2.5 to 12 mM. Results represent the mean ± S.E. for three independent experiments. *, p < 0.005 versus Ad-siControl.

FIGURE 6. siRNA-mediated suppression of PC increases the pool of acetyl carnitines in 832/13 cells. Cellular levels of acetyl carnitine in Ad-siControl- and Ad-siPC-treated cells were measured by quantitative tandem mass spectrometry. Data are corrected for protein content and expressed relative to levels in Ad-siControl-treated cells. Each value represents the mean ± S.E. for three independent experiments. *, p < 0.001.

FIGURE 7. siRNA-mediated suppression of PC has no effect on NADPH-to-NADP ratio in 832/13 cells. After treatment with Ad-siPC or Ad-siControl, cellular NADPH and NADP levels were measured using an enzymatic cycling assay and expressed as NADPH:NADP ratio. Data represent the mean ± S.E. for three independent experiments.

FIGURE 8. siRNA-mediated suppression of PC decreases ATP and αKG levels in 832/13 cells. Total ATP levels in cells treated with 12 mM glucose (A) and tricarboxylic acid (B) cycle intermediate levels at low and high glucose were measured in 832/13 cells pretreated with Ad-siPC or Ad-siControl using a luciferase kit and a quantitative GS/MS method, respectively. Data represent the mean ± S.E. for three independent experiments. *, p < 0.05.
β-cells to palmitate was reported to reduce PC (36), although others observed no change in PC levels in islets exposed to fatty acids (37). Furthermore, it was reported that the specific activity of PC is increased in extracts of islets from insulin-resistant, prediabetic Zucker fatty rats, suggesting a means by which pyruvate cycling activity and insulin secretion might increase to compensate for insulin resistance (38). Although these prior observations were based on static measurements of enzyme activities in cell extracts, a recent study provided a direct measurement of the effects of chronically elevated free fatty acid levels on pyruvate cycling activity in living cells and demonstrated that lipid-induced impairment of GSIS was accompanied by ablation of the normal glucose-induced increment in pyruvate cycling activity due to a large increase in cycling at basal glucose levels (8).

Given the seemingly critical role of PC in anaplerotic metabolism of pyruvate and pyruvate cycling activity, the current study focused on application of siRNA technology for manipulation of PC expression and evaluation of the effects of these maneuvers on GSIS and metabolic activities in β-cell lines and primary rat islets. Treatment of 832/13 cells with Ad-siPC reduced PC mRNA levels by 83 ± 1% and PC protein by 56 ± 2% without affecting cell viability or growth rate (not shown). Strikingly, this manipulation did not result in impairment of GSIS but rather in a modest but significant increase in insulin secretion at stimulatory glucose. Similarly, GSIS was not impaired in response to Ad-siPC treatment of primary rat islets under conditions in which PC mRNA and protein were suppressed by 64 ± 3% and 35 ± 5%, respectively.

To obtain a better understanding of these findings, we performed extensive metabolic analyses of cells with and without suppression of PC expression, including radioisotopic and 13C NMR-based measurements of metabolic flux and MS-based metabolic profiling experiments. We found that suppression of PC expression did not change glucose usage or oxidation in 832/13 cells. Importantly, an average 56% decrease in PC protein levels resulted in only a 20 ± 3% reduction in flux through PC at stimulatory glucose and no significant difference in the increment in pyruvate cycling activity as glucose was raised from 2.5 to 12 mM. Given that only a single isoform of PC has been described, these results suggest a compensatory increase in PC-specific activity in response to maneuvers that decrease its expression.

Acetyl-CoA allosterically activates PC in most species, including rats, from which the INS-1-derived cell lines and primary islets used in this study were derived (reviewed in Ref. 27). Here, we found that acetyl carnitine, a metabolite in close equilibrium with acetyl-CoA, is increased by 36 ± 6% in 832/13 cells with decreased PC expression. Metabolic profiling studies further demonstrated a rise in intracellular lactate levels, with no change in pyruvate in Ad-siPC-treated cells. Taken together, these data suggest that the 20% decrease in flux through PC was sufficient to trigger alternative pathways of pyruvate metabolism, such as conversion to lactate via lactate dehydrogenase and conversion to acetyl-CoA via pyruvate dehydrogenase, thus explaining the accumulation of lactate and acetyl-CoA. The rise in acetyl-CoA suggests a mechanism by which a compensatory increase in PC activity may have occurred in response to lowering of PC protein levels, thus explaining the disparity between the 56% reduction in PC protein levels in Ad-siPC-treated cells and the lesser decrease (20%) in PC flux.

The 13C NMR-based method for measuring pyruvate cycling quantifies flux through the combined carboxylation (PC) and decarboxylation (cytosolic or mitochondrial malic enzyme) reactions associated with pyruvate cycling but does not precisely identify the pathways that contribute to this cycle. As
shown in Fig. 9, pyruvate cycling can occur via the pyruvate/malate, pyruvate/citrate, or pyruvate/isocitrate shuttles. The pyruvate/isocitrate shuttle refers to citrate leaving the mitochondria either as citrate or as isocitrate and isocitrate being oxidized to αKG via cytosolic, NADP-dependent isocitrate dehydrogenase. αKG, in turn, re-enters the mitochondria for recycling to pyruvate via malic enzyme. We have recently demonstrated the importance of the pyruvate/isocitrate shuttle in regulation of GSIS. First, siRNA-mediated silencing of the citrate/isocitrate carrier resulted in a dramatic decrease in GSIS and a corresponding reduction of the NADPH/NADP ratio (15). In addition, treatment of β-cells with an siRNA that specifically targets the cytosolic, NADP-dependent isocitrate dehydrogenase caused impairment of GSIS and a decrease in pyruvate cycling flux and NADPH:NADP ratio (15). Both observations are consistent with the recently proposed role of NADPH as a coupling factor in GSIS (12, 13). Also aligning with this idea, Ad-siPC administration had no effect on the NADPH:NADP ratio during glucose stimulation, thereby possibly explaining the lack of effect of PC silencing on GSIS. However, our results are also in accord with a recently proposed alternative model in which other by-products of cytosolic αKG metabolism could be key signaling molecules for GSIS (39).

How do cells with reduced PC expression manage to keep NADPH levels intact despite lower pyruvate cycling flux? A potential clue comes from the observation that cells with lower PC expression have reduced cellular ATP levels in the face of unchanged glucose oxidation. Simultaneously, αKG levels are significantly decreased by Ad-siPC treatment. We propose that Ad-siPC-treated cells compensate for reduced PC flux via one or a combination of the following mechanisms. 1) Maintenance of NADPH production via increased flux through the NADP-dependent cytosolic, NADP-dependent isocitrate dehydrogenase at the expense of the corresponding NADH-producing steps in the mitochondria (e.g. isocitrate is preferentially converted to αKG by cytosolic, NADP-linked ICD rather than mitochondrial, NAD-linked ICD). This flux alteration will not change glucose oxidation as measured by 14CO2 release but is likely to change cellular ATP levels due to lower NADPH production. 2) Consumption of αKG in the cytosol to produce a metabolite that is critical for GSIS. In this regard, αKG can be converted to glutamate, which has been implicated in GSIS signaling (40, 41), although some other laboratories have failed to confirm these findings (42–44). In light of a recent publication implicating metabolites downstream of αKG in GSIS (39), this issue is deserving of further investigation.

Interestingly, unlike the genetic maneuvers described in this report, administration of a pharmacologic inhibitor of PC, phenylacetic acid, has been shown to cause significant inhibition of GSIS (9, 10) as well as inhibiting PC flux and pyruvate cycling activity (5). This seemingly discordant finding is likely explained by the fact that the CoA form of phenylacetic acid impairs the allosteric activation of PC by acetyl-CoA, thereby interfering with a key step in compensation against PC suppression (45).

In sum, our studies have demonstrated that β-cells possess compensatory systems that are activated in response to suppression of PC enzyme levels. The compensatory events include allosteric activation of PC due to increased levels of acetyl-CoA and enhanced conversion of isocitrate to αKG and downstream metabolites via the cytosolic, NADP-dependent isocitrate dehydrogenase. The presence of such a system suggests that β-cells are able to “defend” against suppression of anaplerosis and pyruvate cycling pathways. Elements of these compensatory responses may be involved in the remarkable ability of β-cells to respond to diet-induced obesity. Thus, consumption of high-fat diets by many strains of rodents or in humans leads to severe insulin resistance but generally not to β-cell failure unless age or genetic susceptibility factors are present. Further understanding of these mechanisms may lead to new therapeutic strategies that seek to mimic or encourage key elements of the compensatory system.

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