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The role of pyruvate carboxylase in insulin secretion and proliferation in rat pancreatic beta-cells *

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Abstract

Aims/hypothesis—Pyruvate carboxylase (PC) or pyruvate dehydrogenase (PDH) is required to transfer carbons from pyruvate into the Krebs cycle. PC activity is preserved in the islets of obese animals but it is reduced in the islets of type 2 diabetic animals, suggesting that PC is important in beta-cell adaptation to insulin resistance and that PC reduction may lead to beta-cell failure.

Methods—To confirm the significance of PC, first we lowered activity using PC siRNA in INS-1 cells and in dispersed rat islet cells; second, we overexpressed PC in INS-1 cells; third, we inhibited PDH by overexpressing pyruvate dehydrogenase kinase 4 (PDK4) in INS-1 cells.

Results—Treatment of INS-1 cells or dispersed rat islet cells with PC siRNA resulted in a significant reduction in insulin secretion in both cell types and reduced proliferation in INS-1 cells, this treatment also reduced the contents of oxaloacetate, malate, ATP, and the NADPH/$NADP^+$ ratio and reduced activity of the PMS; overexpression of PC in INS-1 cells led to an elevation of insulin secretion and cell proliferation; inhibition of PDH activity by overexpressing PDK4 in INS-1 cells did not reduce insulin secretion.

Conclusions/interpretation—Our findings indicate that the PC pathway in beta-cell might play a key role in pyruvate metabolism, insulin secretion and cell proliferation.

Keywords

Pyruvate carboxylase; pyruvate dehydrogenase; insulin secretion; pyruvate-malate shuttle; islet of Langerhans; INS-1 cell

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The authors declare that there is no duality of interest associated with this manuscript.

Introduction

In addition to the firmly established role of glycolysis in regulation of glucose stimulated insulin secretion (GSIS), the mitochondrial component of glucose metabolism has also been implicated in pancreatic beta-cell function. There are two pathways by which pyruvate can enter the mitochondrial Krebs cycle, catalyzed by pyruvate carboxylase (PC) (1;2) and pyruvate dehydrogenase (PDH) (3;4). Pyruvate is converted to the Krebs cycle intermediate oxaloacetate (OAA) by PC and to acetyl-CoA by PDH (5). In most cells the PDH pathway predominates. However, pancreatic beta-cells express unusually high levels of PC (6). As a result, the beta-cell is unique in that approximately equal amounts of pyruvate enter into Krebs cycle via PC and PDH (7). A primary role of PC in tissues such as liver and kidney is to provide substrate for gluconeogenesis. However, as pointed out by MacDonald (8), beta-cells lack the essential gluconeogenic enzyme phosphoenolpyruvate carboxykinase, and therefore the high levels of PC must be required for a different function in beta-cells. PC is essential for anaplerosis, which is needed to maintain the Krebs cycle. Krebs cycle intermediates are depleted by many processes including lipogenesis and synthesis of certain amino acids (9; 10). PC is able to restore the lost intermediates by producing OAA from pyruvate. OAA can be converted to malate by malate dehydrogenase, then malate is released to the cytosol where it is converted back to pyruvate by malic enzyme to form the pyruvate-malate shuttle (PMS) (1) or pyruvate cycling (11;12). This shuttle requires the activity of PC and in beta-cells it is capable of producing more NADPH than the pentose phosphate shuttle (8).

A number of investigators have provided evidence that mitochondrial metabolism is coupled to insulin secretion through PC rather than PDH. Inhibition of PC with phenylacetic acid prevents GSIS (13–16). ^{13}C-NMR isotopomer analysis (11;12) has shown a close correlation between flux through PC and the capacity of glucose to stimulate secretion. Entry of pyruvate into the Krebs cycle via PDH does not appear to be important since activation of PDH had only a minor effect on glucose metabolism and insulin release (17). In addition, a recent report (18) found that a single nucleotide polymorphism in the PC gene of African Americans is significantly associated with the magnitude of the acute insulin response.

Type 2 diabetes is characterized by insulin resistance and beta-cell failure (19). Once insulin resistance occurs, pancreatic beta-cells must secrete more insulin to maintain normal glucose levels. Increased insulin secretion requires beta-cell adaptation, a process that includes both enhanced insulin secretion and increased beta-cell proliferation (20). In type 2 diabetes, the failure of beta-cell adaptation coincides with reduced activities of PC (1;2) and PDH (3;4). We have provided evidence that PC is more important to beta-cell adaptation than PDH (13;14; 21;22). During compensated obesity, islet PDH but not PC is reduced (13;14), indicating that normal PDH activity is not required for beta-cell adaptation. In addition, the PC inhibitor phenylacetic acid reduces the beta-cell proliferation response in 60% panreatectomized rats (14) and in Zucker fatty rats (13).

Current evidence for a key role of PC in beta-cell function is extensive but is limited to correlation or it is dependent on the relatively nonspecific PC inhibitor phenylacetic acid. Recently Newgard's group has provided more evidence to support the importance of PC in beta-cells (23;24). In the present study we performed specific genetic manipulation of PC activity up or down to produce corresponding changes in beta-cell proliferation and insulin secretion; effect of PDH inhibition on insulin secretion was also tested.

Subjects, materials and methods

Animals

The principles of animal laboratory care under the guidelines of both NIH and the University of Louisville's Animal Care Committee were followed strictly. Male Sprague-Dawley (SD) rats (Taconic, Germantown, NY) weighting 120–150g were used for this research.

Cell culture

Rat insulinoma INS-1 cell line was provided by Dr. G.H. Hockerman (Purdue University), and the cells (passage from 40 to 60) were cultured as described previously (25).

PC siRNA sequence design

PC gene siRNA and control sequence were designed and synthesized by Invitrogen (see Table 1 legend).

PC siRNA transfection

PC siRNA transfection was carried out according to Stealth RNA transfection protocol (Invitrogen), and the cells were cultured for up to 72 hours.

Overexpression of PC and PDK4 in INS-1 cells

Human PC cDNA was provided by Mary Anna Carbone in the laboratory of Dr. Brian H. Robinson at the University of Toronto. Mouse pyruvate carboxylase kinase 4 (PDK4) cDNA was a gift from Dr. Nam Ho Jeoung in the laboratory of Dr. Robert A. Harris at the Indiana University. PC or PDK4 gene was cloned into EcoRI site of pCDNA3 (Invitrogen) driven by CMV promoter. Transfection of these genes into INS-1 cells was carried out according to the instruction of Lipofectamine 2000 (Invitrogen). Cell clones with stable PC or PDK4 over-expression were selected using G418. Control cells were treated with the vector that contains neither PC nor PDK4 gene; all other treatments were same as PC or PDK4 treated cells. The experiments were carried out on an isolated clone with the highest overexpressed PC or PDK4.

Measurements of protein and DNA contents

Protein content was assayed by a commercial kit that used BSA as standard (Bio-Rad, Hercules, CA), and cell DNA content was determined using PicoGreen dsDNA Quantitation kit (Molecular Probe).

PC activity assay

PC activity was measured according to the method of MacDonald et al (1) using [^{14}C] $NaHCO_3$.as a substrate.

Mitochondria isolation and PC protein detection

Islet mitochondria isolation and detection of PC protein were measured using the method of MacDonald et al. (6) with modification (26).

Active PDH activity assay

Active PDH was measured as described previously (4;27). Cell homogenates were used for the PDH activity assay.

[^{3}H]Thymidine incorporation

[^{3}H]Thymidine incorporation assay was performed by a previously described method (28), and the radioactivity in the membranes was counted by a scintillation counter.

Islet isolation and separation of islets into single cells, and PC siRNA transfection

The islets were isolated from 10 weeks old male Sprague-Dawley (SD) rats by an adaptation of the Gotoh method (29). Islets were digested with 0.25% trypsin for 5 min, uncompleted digested islets were separated into single cells by passing through a 200 μl pipette tip. Dispersed islet cells were cultured for 1 hour at 37°C in RPMI 1640, and treated these cells with PC siRNA for 48 hours as described in *PC siRNA transfection* section.

Insulin secretion and insulin assay

Insulin secretion was performed by a previously described method (21). Insulin secreted into KRB buffer was measured using an ELISA insulin assay kit.

Glucose utilization and oxidation

Glucose utilization was measured by the method of Ashcroft (30), glucose oxidation was measured by the method of Kosokawa (31).

NADPH and NADP$^+$ measurement

The cells or islets (~40 μg protein) were homogenized in 50 μl of buffer that contained 10 mmol/l Tris, pH 8.1, 1% NP-40, 0.5 mmol/l PMSF, and 0.5% Triton X-100. After standing on ice for 20 min, the homogenate was centrifuged for 2 min at 12,000 × g. The supernatant was combined with 5 μl of 2 mol/l NaOH to destroy NADP$^+$ (for NADPH assay) or 5 μl of 2 mol/l HCl to destroy NADPH (for NADP$^+$ assay) at room temperature for 30 min followed by adding 5 μl of 2 mol/l HCl or 5 μl of 2 mol/l NaOH to neutralize pH to ~7. NADPH or NADP$^+$ contents in the extract were measured by a modified method based on a cycling method described by Lower and Passonneau (32). One hundred μl of cycling reagent that contained 100 mmol/l Tris, 80 mmol/l HCl, 9 U/ml of type II glutamate dehydrogenase, 5 mmol/l *a*-ketoglutaric acid, 2 mmol/l glucose 6-phosphate, 0.1 mmol/l ADP, 10 mmol/l ammonium acetate, 0.1% BSA, and 6 U/ml glucose-6-phosphate dehydrogenase was added to each tube and then incubated for 60 min at 38 °C, followed by heating to 100 °C for 10 min to stop the reaction. After brief centrifugation, a 100 μl of sample was transferred to a UV cuvette that contained 0.9 ml reaction buffer having 0.006 U/ml 6-phosphogluconate dehydrogenase, 30 μmol/l NADP$^+$, 0.1 mmol/l EDTA, 30 mmol/l ammonium acetate, and 5 mmol/l $MgCl_2$ and stood at room temperature for 30 min. The formed NADPH was measured by a fluorometer at 340 nm excitation and 420 nm emission. NADPH and NADP$^+$ standards (0–10 nmol) were run in parallel through the entire procedure, including the extraction steps. The NADPH/NADP$^+$ ratios were calculated from the measured NADPH and NADP$^+$ contents in each assay. The blank was obtained from reaction buffer that contained islet extract but did not contain glucose-6-phosphate dehydrogenase and glutamate dehydrogenase.

ATP, OAA and malate content measurement and malate release from isolated mitochondria

Cell extract preparation and ATP content measurement were performed using a commercial kit (Molecular Probes). Oxaloacetate (OAA) and malate contents were measured from cell PCA extract by the methods of Parvin et al. (33). The method for malate release from isolated mitochondria is previously described (22).

Data presentation and statistical methods

All data are expressed as mean ± S.E.M. The listed *n* values represent the number of an individual experiment performed. Comparisons between two groups were performed by Student's *t* test. Comparisons between multiple groups were performed by one- or two-way ANOVA (Tukey post hoc test). A value of $p<0.05$ was considered significant.

Results

PC siRNA significantly decreased PC activity and protein concentration in treated INS-1 cells

We used siRNA to reduce PC protein and activity in INS-1 cells. In our preliminary tests, we compared the inhibitory effects of three pairs of siRNA on PC activity and GSIS (data not shown), and one of them with the strongest effects (Table 1) was chosen for this study. As shown in Figure 1A, siRNA significantly decreased PC activity (45% of control, $p<0.01$) 48 hours after treatment. We chose 48h of siRNA treatment because inhibition of PC activity in preliminary studies was greatest at 48 h (data not shown). An inserted picture in Figure 1A is a representative Western blot that shows that content of the 130 kDa PC protein was reduced. Quantification of the data is shown under the protein bands. The values of relative optical density (normalized to actin) were significantly reduced in the PC siRNA treated group ($p<0.05$).

PC siRNA significantly reduced GSIS in INS-1 cells

To test if PC regulates insulin secretion, we inhibited PC activity by treating INS-1 cells with PC small interfering RNA (siRNA) for 48 hours. The medium was then changed to Krebs-Ringer buffer, and GSIS was performed in the presence of 2.8, 5.5 and 16.7 mmol/l glucose. Figure 1B shows that PC siRNA significantly down-regulated the glucose dose-response curve of insulin secretion in INS-1 cells. PC siRNA, however, could not reduce KCl induced insulin secretion (Figure 1C); suggesting the regulatory mechanism by KCl of insulin secretion is different from the PC pathway.

PC siRNA significantly suppressed DNA and protein synthesis

We previously reported that changes in PC activity were associated with changes in beta-cell proliferation (13;14). To test if this was a causal relationship we measured ^{3}H-thymidine incorporation and protein content in PC siRNA treated INS-1 cells. The result shown in Figure 1D demonstrates that ^{3}H-thymidine incorporation in 48 h PC siRNA treated INS-1 cells was decreased to 78% of control ($p<0.05$). Parallel changes were seen in cell protein content in PC siRNA treated cells (Figure 1E). Our data demonstrate that inhibition of PC by siRNA leads to a reduction in DNA and protein synthesis, which are parameters for cell proliferation.

Inhibition of PC did not reduce glucose utilization and oxidation in INS-1 cells

We expected that inhibition of PC would lead to a reduction in glucose oxidation because about 50% of beta-cell pyruvate enters the Krebs cycle by the PC pathway (7). To test this, we measured glucose utilization and oxidation in 48 h PC siRNA treated INS-1 cells. Surprisingly, glucose utilization and oxidation were not reduced (Figure 2A and B). This result is consistent with that reported from Frannson et al. (16); they used phenylacetic acid (PAA, 5 mmol/l) to inhibit PC in isolated rat islets, and their results showed that PAA could not inhibit glucose oxidation. We then measured PDH activity in these INS-1 cells. PDH activity was 2.7-fold increased (Figure 2C, $p<0.01$ vs. control). Interestingly, the increase in PDH does not compensate for the effect of reduced PC on GSIS and or beta-cell proliferation (Figure 1). The increase in PDH activity may be due to an increase in pyruvate concentration or a decrease in $NADH/NAD^{+}$ ratio and it may not be associated with a change in flux; this would explain the lack of change in glucose oxidation.

PC siRNA significantly reduced GSIS in dispersed islet cells

Because INS-1 cells are a tumor cell line, we tested if PC siRNA regulates GSIS in primary beta-cells. The islets isolated from male SD rats were dispersed to single cells in order to improve the efficacy of siRNA treatment. Dispersed islet cells are less responsive to glucose (34) but dispersion is necessary for all cells to be exposed to the siRNA. As shown in Figure 3A we achieved a significant reduction in PC activity. Importantly, PC inhibition reduced GSIS significantly (Figure 3B) when cells were stimulated with a high concentration of glucose (16.7 mM). Figure 3B shows that glucose responsiveness in control group was poor. To know the reasons, we have tested the effect of transfection medium on both primary beta-cells and INS-1 cells. Our data showed that GSIS in primary beta-cells was significantly suppressed by the transfection medium, but INS-1 cells were less affected (data not shown); suggesting chemical toxicity from the transfection medium and vulnerability in primary beta-cells.

Intermediates and products of PMS and ATP content were reduced in PC siRNA treated beta-cells

Evidence that the PMS is related to insulin secretion (11;14) prompted us to measure OAA, malate contents and NADPH/$NADP^+$ ratio since they are the intermediates or products of this shuttle. As shown in Table 2, all of these parameters were significantly reduced in PC siRNA treated INS-1 cells and dispersed islet cells. NADPH/$NADP^+$ ratios were significantly increased in response to high glucose (16.7 mM) in both control and siRNA treated INS-1 cells and primary beta-cells, and this increase was inhibited by siRNA treatment or enhanced by PC overexpression. To test shuttle activity, we measured malate release from mitochondria isolated from PC siRNA treated dispersed islet cells and INS-1 cells. Malate release from PC siRNA treated INS-1 cells (Table 2) and dispersed islet cells (Figure 3C) was significantly reduced. These results confirm that PC controls flux through the PMS and that reductions in PC and shuttle activity coincide with reductions in insulin secretion and cell proliferation. Fransson et al. (16) have reported that PC is required for the fuel-induced rise in the ATP:ADP ratio, thus we measured ATP contents in PC siRNA treated INS-1 cells. Our result shown in Table 2 indicates that PC siRNA significantly reduced ATP contents in these cells.

Elevation of PC significantly up-regulated insulin secretion and cell proliferation in stable PC overexpressing INS-1 cells

Our results from PC siRNA studies demonstrated that inhibition of PC reduced insulin secretion (Figure 1B and Figure 3B) and cell proliferation (Figure 1C and D). If elevation of PC leads to the opposite results, this would further support our hypothesis that PC has strong control in beta-cell function. Therefore we overexpressed PC in INS-1 cells. The clone with the highest level of PC overexpression was obtained and used in present study. PC activity in this clone was increased about 2-fold (Figure 4A); insulin secretion was increased 2.5-fold at 2.8 mmol/l glucose and 3-fold at 16.7 mmol/l glucose (Figure 4B). Basal insulin secretion was also slightly but significantly increased in other lower level PC overexpresing clones (data not shown); suggesting that PC also controls basal insulin secretion. The stimulatory effect of PC overexpression on basal insulin secretion (Figure 4B, 2.8 mmol/l glucose) agrees with the inhibitory effect of PC siRNA on basal insulin secretion (Figure 1B). DNA and protein synthesis was significantly increased (Figure 4C and D). Contents of OAA and malate, NADPH/$NADP^+$ ratio, and malate release from mitochondria were also significantly elevated in PC over-expressing INS-1 cells (Table 2). All of these results demonstrate that PC plays a key role in insulin secretion and cell proliferation.

Reduction of PDH by PDK4 overexpression did not reduce insulin secretion

PDH activity was increased in PC siRNA treated INS-1 cells (Figure 2C). Despite the increase in PDH activity, GSIS was reduced (Figure 1B). This indicates that the PDH pathway may not

be important for insulin secretion. To further test this we developed INS-1 cells with reduced PDH activity. This was accomplished by taking advantage of the fact that PDH activity is inactivated by pyruvate dehydrogenase kinase (PDK) through phosphorylation (35). There are four PDK isoforms in pancreatic islets (27;36). We reduced PDH pathway by over-expressing the isoform PDK4 in INS-1 cells. The clone with the highest level of PDK4 overexpression was obtained and used in present study. PDK-4 over-expression in this clone produced a great reduction in PDH activity (Figure 5A) but no significant change in PC activity (Figure 5B). As shown in Figure 5C, reduced PDH activity in INS-1 cells did not significantly change GSIS. Preliminary tests of glucose oxidation in PDK-4 over-expressing INS-1 cells indicated that [U-^{14}C]glucose oxidation was not significantly changed (data not shown).

Discussion

Glucose metabolism regulates insulin secretion (37–39) and blocking glucose phosphorylation (40) or glycolysis (37) inhibits insulin secretion. Of the many enzymes and intermediates needed to complete glycolysis and glucose oxidation, it is uncertain which plays a key role in the regulation of insulin secretion. Candidate enzymes include PC and PDH which in pancreatic beta-cells, metabolize equal amounts of pyruvate into the Krebs cycle (7). Previous studies (11;12;18) including those from our group (13;14) suggested that PC might be important for insulin secretion and cell proliferation; however, none of those reports provided direct, causative evidence to confirm this hypothesis. In the current study, we performed the following specific genetic manipulations to test the role of PC versus PDH: 1) PC activity was decreased with siRNA in INS-1 cells and dispersed islet cells, 2) PC activity was increased by PC gene overexpression and 3) PDH activity was reduced by PDK4 overexpression in INS-1 cells. By PC gene manipulation, we demonstrated that reduction or elevation of PC activity caused corresponding changes in insulin secretion and cell proliferation. In contrast, PDH inhibition did not reduce insulin secretion. The inhibition of PC expression by siRNA produced a remarkable elevation in PDH activity that could substitute for impaired PC activity in order to maintain normal rates of glucose oxidation. But elevated PDH could not compensate for the affect of diminished PC to reduce insulin secretion. This means that essential role of PC does not stem from maintaining glucose oxidation, but rather the critical capacity of PC must be to feed substrate into analplerosis and the PMS.

The portion of the current study that involved siRNA inhibition of the PC gene was also recently carried out by Jensen et al (23) in the Newgard laboratory. Parts of their results were consistent with our findings, while other results differed markedly: Similar to our data, they found that inhibition of PC (50% down-regulation of PC) had no direct affect on either glucose utilization or oxidation. Also, they (23) found that inhibition of PC reduced PMS, measured in our studies by malate release from mitochondria and in their studies by via ^{13}C NMR isotopomer analysis. On the other hand, the two studies differed significantly in the effects of PC inhibition on beta-cell content of NADPH or malate and on the effect of PC inhibition on insulin secretion. For explaining insulin secretion result, they performed radioisotopic and ^{13}C-NMR-based measurements of metabolic flux and MS-based metabolic profiling experiments; they found that an average 56% decrease in PC protein levels resulted in only about 20% reduction in flux through PC at stimulatory glucose and no significant difference in the increment in pyruvate cycling activity when glucose concentration was increased. They also found that acetyl-CoA and lactate levels are increased in INS-1 cells with decreased PC expression, suggesting a compensatory mechanism in these cells. They also believed PC inhibition can be compensated by pyruvate/isocitrate shuttle. These are the explanations for their insulin secretion data. In our study inhibition of PC depleted both malate and NADPH. We expected this since beta-cell PC is a major source of malate and the malate-pyruvate shuttle is a major source of NADPH (8). As there are large reductions in NADPH/NADP$^+$ ratios and Krebs cycle intermediates in siRNA treated INS-1 cells (Table 2), it is possible that this contributes to the reduction in DNA

synthesis and protein content (Figure 1C and D). In contrast to our results, Jensen et al (23) found no effect of PC inhibition on either malate content or NADPH.

We are confident in our findings, since we not only found that PC inhibition depleted NADPH and malate content, but we also found that elevation of PC produced a corresponding increase in NADPH and malate contents. The other difference in the two studies is that we found insulin secretion to be sensitive to increased or decreased PC activity while Jensen et al (23) saw no impact of PC inhibition on GSIS. It is possible that the differences in results are due to the differences in the INS-1 cell lines used by Jensen and by us. Different strains of INS-1 cells display very different characteristics for insulin secretion (41) and PMS (11). For example, malate content at similar glucose concentrations was more than twice as high in Jensen's cells than our own. Also GSIS was many fold greater in Jensen's cells. These differences could have been due to higher basal levels of PC activity in Jensen's cells, which might be expected to reduce sensitivity to siRNA inhibition. However, the PC activity of Jensen's cells is not known and the reason for the different findings remains uncertain. Very recently, this group provided additional evidence to support an important role of the PMS in the regulation of GSIS (24). This is consistent with our conclusion.

The importance of the PC pathway for beta-cell proliferation, a response essential to the beta-cell's adaptive response to insulin resistance, was demonstrated in the current study. Conversion of pyruvate to OAA, then to malate and back to pyruvate generates three important intermediates for cell proliferation; OAA, NADPH and malate. OAA is used for protein and lipid synthesis (42–44), NADPH is needed for lipid and fatty acids synthesis (43;44) and insulin secretion (8;45) and malate is used for production of NADPH. Previous studies have suggested an important role for PC in beta-cell proliferation: We reported that there is more than a 2-fold increase in PC activity in islets from insulin resistant, obese Zucker rats that have a 3.9-fold (13) increase in islet beta-cell mass. Cline et al (12) reported that a ~4 fold increase in anaplerosis, which provides substrate for proliferation, could be attributed to an increase in PC flux in INS-1 beta-cells. We found that phenylacetic acid inhibition of PC prevents the full beta-cell proliferative response to 60% pancreatectomy (14). In addition, a proliferative role for PC is not unique to the beta-cell. Mutations to PC in yeast cells inhibits growth (46;47), and PC activity is elevated in growing tumor cell populations (48). The current study provides the most specific and direct evidence for PC's role in beta-cell proliferation.

In summary, by manipulating PC and PDH expression levels we have provided the most direct evidence that the PC pathway plays a key role in insulin secretion in dispersed islet cells and INS-1 cells, and in cell proliferation in INS-1 cells. Since the effects of PC were independent of total glucose metabolism and since the PDH pathway is much less important than PC, we suggest that it is the role of PC in anaplerosis and the PMS pathway that makes it so important for insulin secretion.

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Abbreviations

PC, pyruvate carboxylase; siRNA, small interfering RNA; PDH, pyruvate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; PMS, pyruvate-malate shuttle; GSIS, glucose stimulated insulin secretion.

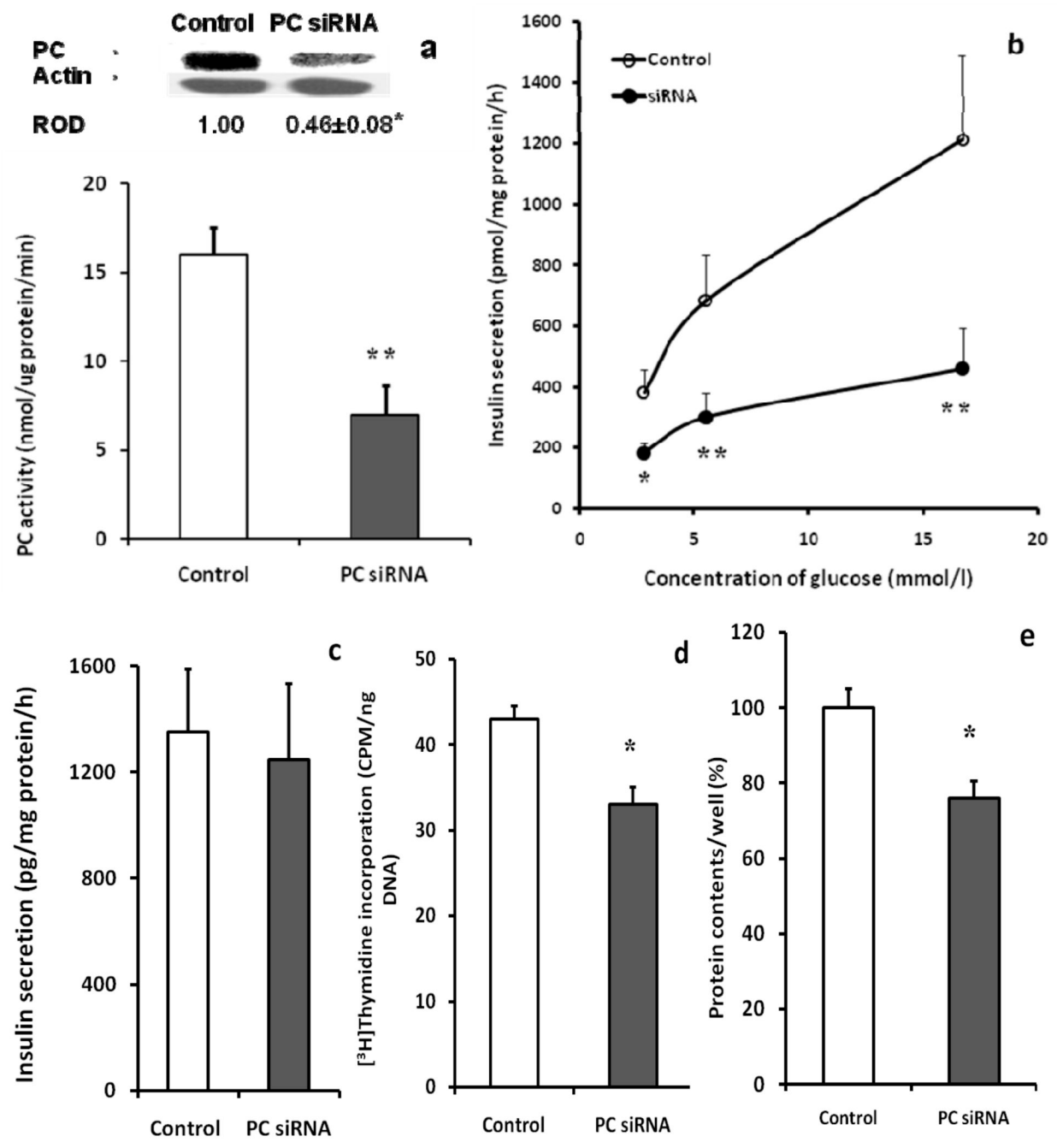

Figure 1.

PC siRNA decreases PC protein content, insulin secretion, cell proliferation and protein content in 48 h treated INS-1 cells. A, PC activity and PC protein content. The insert picture shows a representative Western blot (total 3) of PC protein (~130kDa) and actin in control and PC siRNA treated cells. The numbers under the protein bands indicate the data of protein band quantification (n=3). ROD means relative optical density after normalization to actin. B, glucose (2.8, 5.5 and 16.7 mmol/l) stimulated insulin secretion. C, KCl (50 mmol/l) stimulated insulin secretion. D, [^{3}H]Thymidine incorporation. E, total protein content per well. Control means scrambled siRNA treatment. Data are mean ± S.E.M., n=4. * P<0.05, ** P<0.01 vs. controls.

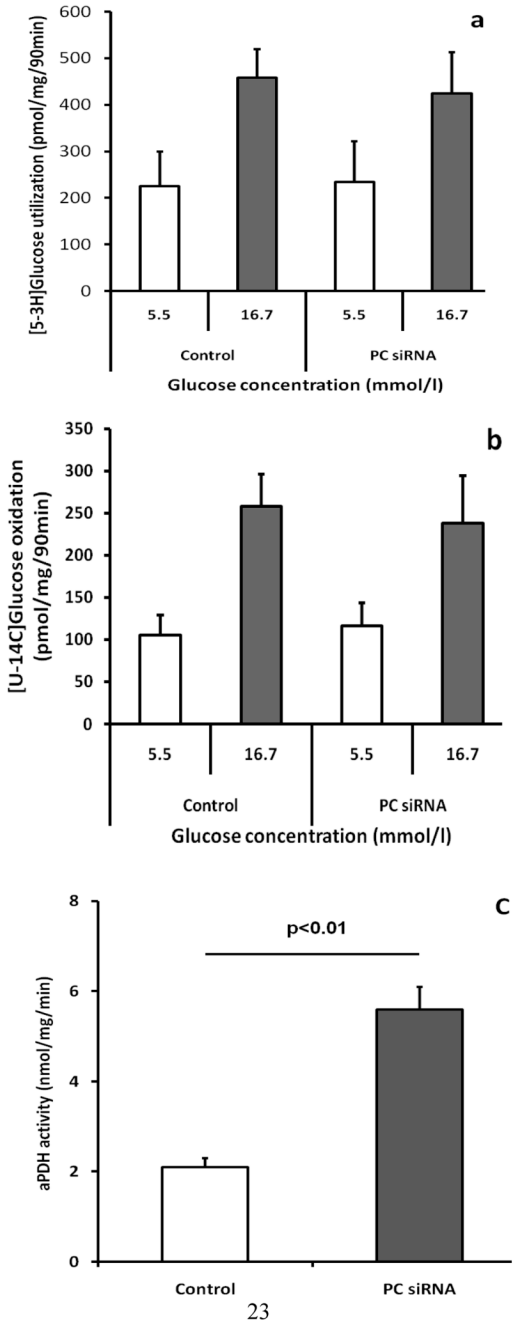

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Figure 2.

Inhibition of INS-1 cell PC with siRNA treatment (48 h) does not change glucose utilization (A) and glucose oxidation (B), but does up-regulate active PDH activity (C) in INS-1 cells. Control means scrambled siRNA. Data are the mean ± S.E.M. n=4.

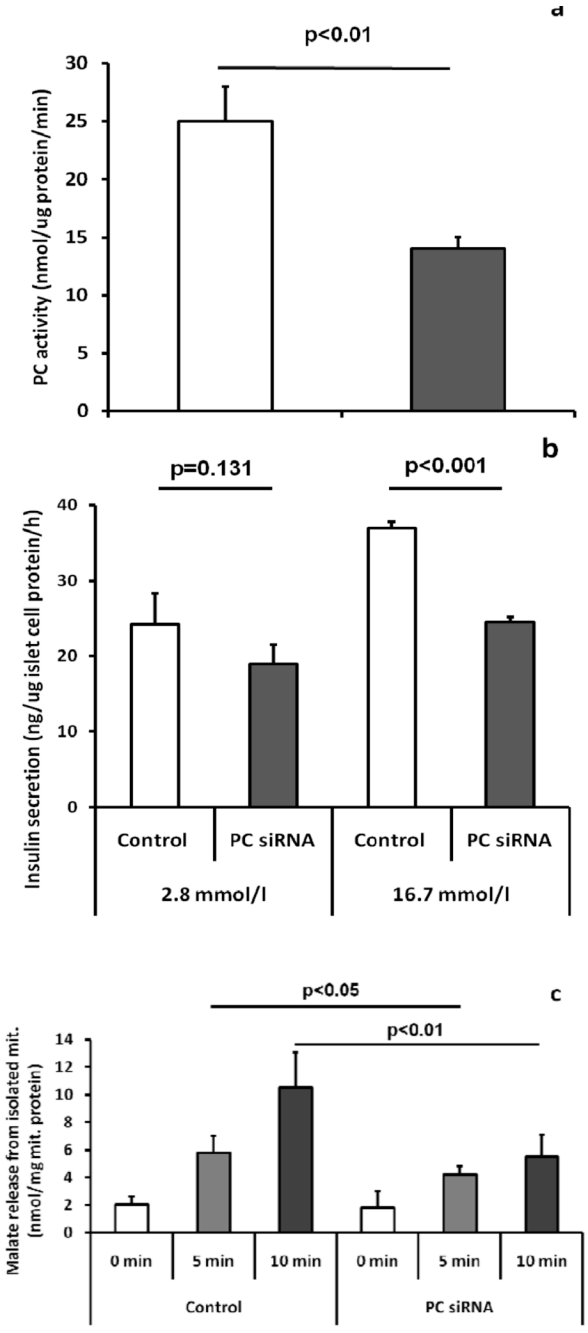

Figure 3.

PC siRNA treatment (48 h) in dispersed primary islet cells reduces PC activity (A) and leads to a reduction in glucose (16.7 mmol/l) stimulated insulin secretion (B) and malate release from isolated mitochondria (C). Control means scrambled siRNA. Data are the mean ± S.E.M. n=4.

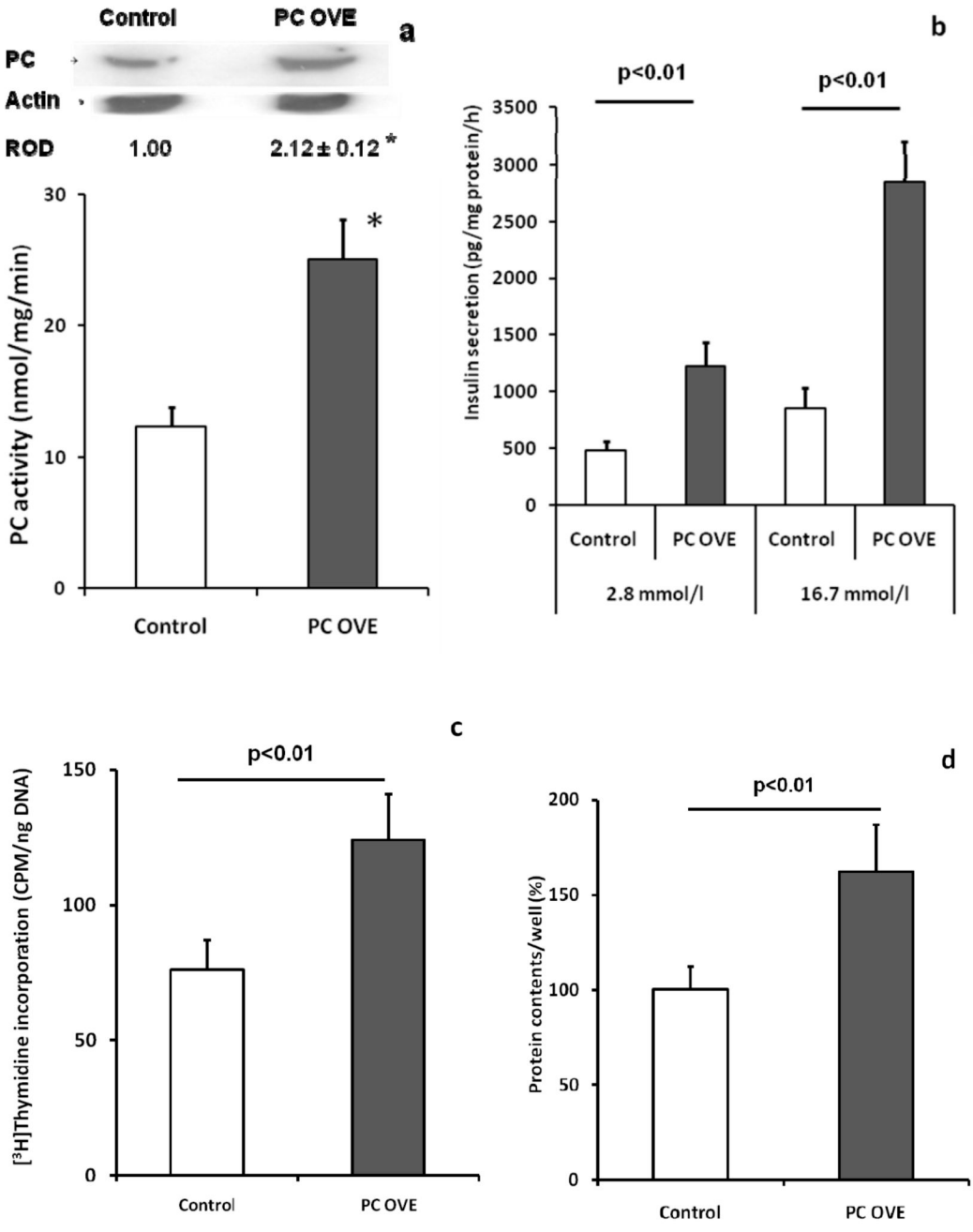

Figure 4.

Stable overexpression of PC in INS-1 cells up-regulates insulin secretion and cell proliferation. A, PC activity and protein content. Western blot of PC protein (~130kDa) and actin shown on the top is a representative experiment (total 3). The numbers under the protein bands indicate the data of protein band quantification (n=3). ROD means relative optical density after normalization to actin. B, glucose (2.8 and 16.7 mmol/l) stimulated insulin secretion; C, [^{3}H] Thymidine incorporation; and D, protein content per well. PC OVE indicates PC over-expressing cells. Control cells were treated with the vector that does not contain PC gene. Data are mean ± S.E.M, n=4. * P<0.05 vs. controls.

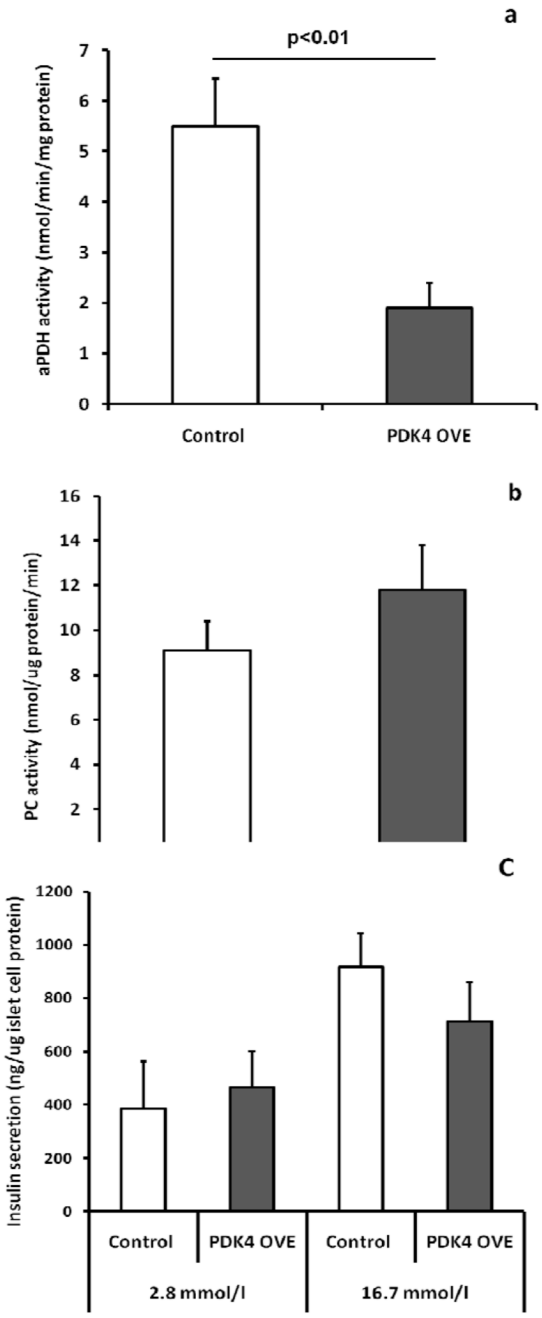

Figure 5.

Reduction of active PDH (aPDH) activity by overexpression (over-exp) of PDK4 (A) does not significantly change PC activity (B) or glucose (2.8 and 16.7 mmol/l) stimulated insulin secretion (C) in INS-1 cells. Control cells were treated with the vector that does not contain PDK4 gene. Data are mean ± S.E.M. n=4.

Table 1

The sequences designed for inhibiting rat PC gene expression in INS-1 cells and dispersed rat islet cells

The PC siRNA and scrambled control siRNA sequences were automatically designed using Invitrogen Stealth RNAi designer on website (www.invitrogen.com), and synthesized by Invitrogen Company.

	Forward primer (5'-3')	Reverse primer (5'-3')
PC siRNA	CCA UCA AGA AAG UAA UGG UGG CCA A	UUG GCC ACC AUU ACU UUC UUG AUG G
Scrambled control siRNA	CCA AGG UGA GAA UGA AGU ACC UCA A	UUG AGG UAC UUC AUU CUC ACC UUG G

Table 2

Metabolite contents in 48 h PC siRNA treated INS-1 cells and dispersed rat islet cells, and in PC over-expressing INS-1 cells

After siRNA transfection, INS-1 cells were cultivated in the culture medium with 11.1 mmol/l glucose, and primary beta-cells with 5.5 mmol/l glucose for 48 hours. All the cells were pre-incubated in KRB containing 0.5% BSA and different concentrations of glucose for 1h, and then the cells were washed with 1x pure PBS for three times before preparation of cell extracts. Concentrations of glucose in pre-cultured medium: 11.1 mmol/l (except indicated in the table) for INS-1 cells and 5.5 mmol/l for dispersed islet cells. mit. means mitochondria. Data are mean ± S.E.M.

Cell types and parameters	Control Scrambled siRNA	PC siRNA	n	P values
INS-1 cells				
OAA, pmol/mg cell protein	177 ± 16	60 ± 14	4	<0.001
Malate, nmol/mg cell protein	7.8 ± 1.9	4.1 ± 1.1	4	=0.028
NADPH/NADP$^+$, pre-cultured with 5.5 mmol/l glucose for 1h	0.35 ± 0.05	0.26 ± 0.07	5	=0.047
NADPH/NADP$^+$, pre-cultured with 16.7 mmol/l glucose for 1h	0.63 ± 0.06 §	0.36 ± 0.13	5	=0.019
Malate release from mitochondria, nmol/10 min/mg mit. protein	12.4 ± 2.3	7.1 ± 1.4	4	=0.013
ATP, pmol/l/mg protein	4.01 ± 0.75	1.78 ± 0.27	4	=0.019
Dispersed SD rat islet cells				
OAA, pmol/mg cell protein	85 ± 13	35 ± 11	4	<0.001
Malate, nmol/mg cell protein	3.2 ± 0.5	1.8 ± 0.2	4	<0.001
NADPH/NADP$^+$, pre-cultured with 5.5 mmol/l glucose for 1h	0.31 ± 0.04	0.24 ± 0.03	5	=0.046
NADPH/NADP$^+$, pre-cultured with 16.7 mmol/l glucose for 1h	0.58 ± 0.15 §	0.32 ± 0.12	5	<0.01
	Control vector	**PC over-expression**		
INS-1 cells				
OAA, pmol/mg cell protein	188 ± 19	293 ± 21	4	<0.001
Malate, nmol/mg cell protein	6.8 ± 1.0	11.0 ± 1.6	4	=0.009
NADPH/NADP$^+$, pre-cultured with 5.5 mmol/l glucose for 1h	0.36 ± 0.08	0.54 ± 0.13	5	<0.05
NADPH/NADP$^+$, pre-cultured with 16.7 mmol/l glucose for 1h	0.61 ± 0.11 §	0.75 ± 0.17 §	5	<0.05
Malate release from mitochondria, nmol/10 min/mg mit. protein	13.4 ± 1.7	20.6 ± 3.2	4	=0.012

§p<0.01 vs. 5.5 mmol/l glucose.