

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 animal models of type 2 diabetes, 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, we first lowered activity using *Pc* (now known as *Pcx*) small interfering RNA (siRNA) in INS-1 cells and in dispersed rat islet cells. Second, we overexpressed *PC* in INS-1 cells, and third, we inhibited PDH by overexpressing the gene encoding 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 content of oxaloacetate, malate and ATP, as well as the NADPH:NADP⁺ ratio and activity of the pyruvate–malate shuttle. Overexpression of *PC* in INS-1 cells led to an elevation of insulin secretion and cell proliferation, whereas 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 cells might play a key role in pyruvate metabolism, insulin secretion and cell proliferation.

Keywords INS-1 cell · Insulin secretion · Islets of Langerhans · Pyruvate carboxylase · Pyruvate dehydrogenase · Pyruvate–malate shuttle

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Abbreviations

GSIS	glucose-stimulated insulin secretion
NMR	nuclear magnetic resonance
PC	pyruvate carboxylase
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PMS	pyruvate–malate shuttle
siRNA	small interfering RNA

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, catalysed by pyruvate carboxylase (PC) [1, 2] and pyruvate dehydrogenase (PDH) [3, 4]. Pyruvate is converted to the Krebs cycle intermediate oxaloacetate by PC and to acetyl-CoA by PDH [5]. In most cells the PDH pathway predominates. However,

pancreatic beta cells produce unusually high levels of PC [6]. As a result, the beta cell is unique in that approximately equal amounts of pyruvate enter into the Krebs cycle via PC and PDH [7]. A primary role of PC in tissues such as liver and kidney is to provide a 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 oxaloacetate from pyruvate. Oxaloacetate 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]. ¹³C nuclear magnetic resonance (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 characterised 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% pancreatectomised 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 is dependent on the relatively non-specific PC inhibitor phenylacetic acid. Recently more evidence was provided 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 downregulation) to produce corresponding changes in beta cell proliferation and insulin secretion; effect of PDH inhibition on insulin secretion was also tested.

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 rats (Taconic, Germantown, NY, USA) weighing 120 to 150 g were used for this research.

Cell culture Rat insulinoma INS-1 cell line was provided by G. H. Hockerman (Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, USA) and the cells (passages 40 to 60) were cultured as described previously [25].

Pc small interfering RNA sequence design *Pc* (now known as *Pcx*) small interfering RNA (siRNA) and control sequence were designed and synthesised by Invitrogen (Carlsbad, CA, USA) (Electronic supplementary material [ESM] Table 1).

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 h.

Overexpression of PC and the gene encoding pyruvate carboxylase kinase 4 in INS-1 cells Human *PC* cDNA was provided by the laboratory of B. H. Robinson (Departments of Biochemistry and Paediatrics, University of Toronto, Toronto, Ontario, Canada). Mouse *Pdk4* cDNA was a gift from the laboratory of R. A. Harris (Departments of Biochemistry and Molecular Biology, Indiana University, Indianapolis, IN, USA). The *PC* or *Pdk4* gene was cloned into EcoRI site of pCDNA3 (Invitrogen) driven by cytomegalovirus promoter. Transfection of these genes into INS-1 cells was carried out according to the manufacturer's instructions for Lipofectamine 2000 (Invitrogen). Cell clones with stable PC or PDK4 overproduction were selected using G418. Control cells were treated with a vector that contained neither the *PC* nor the *Pdk4* gene; all other treatments were the same as for PC- or PDK4-treated cells. The experiments were carried out on an isolated clone with the highest level of overexpression of *PC* or *Pdk4*.

Measurements of protein and DNA contents Protein content was assayed by a commercial kit that used BSA as standard (BioRad, Hercules, CA, USA). Cell DNA content was determined using a quantification kit (PicoGreen dsDNA; Molecular Probes, Eugene, OR, USA).

PC activity assay PC activity was measured according to the method of MacDonald et al. [1] using [^{14}C]NaHCO₃ 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.

[^3H]Thymidine incorporation [^3H]Thymidine incorporation assay was performed by a previously described method [28]. Radioactivity in the membranes was counted by a scintillation counter (Packard Instrument, Ramsey, MN, USA).

Islet isolation and separation of islets into single cells, with subsequent Pc siRNA transfection The islets were isolated from 10-week-old male Sprague–Dawley rats by an adaptation of the Gotoh method [29]. Islets were digested with 0.25% (wt/vol.) trypsin for 5 min and incompletely digested islets were separated into single cells by passing through a 200 μl pipette tip. Dispersed islet cells were cultured for 1 h at 37°C in RPMI 1640 and treated with Pc siRNA for 48 h as described above.

Insulin secretion and insulin assay Insulin secretion was performed by a previously described method [21]. Insulin secreted into KRB was measured using an ELISA insulin assay kit (Linco Research, St. Charles, MO, USA).

Glucose use and oxidation Glucose use was measured by the method of Ashcroft [30]. Glucose oxidation was measured by the method of Hosokawa [31].

NADPH and NADP⁺ measurement The cells or islets (~40 μg protein) were homogenised in 50 μl of buffer containing 10 mmol/l Tris, pH 8.1, 1% (wt/vol.) NP-40, 0.5 mmol/l PMSF and 0.5% (wt/vol.) Triton X-100. After standing on ice for 20 min, the homogenate was centrifuged for 2 min at 12,000 $\times g$. The supernatant fraction was combined at room temperature for 30 min 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), after which 5 μl of 2 mol/l HCl or 5 μl of 2 mol/l NaOH was added to neutralise pH to ~7. NADPH or NADP⁺ contents in the extract were measured by a modified method based on a cycling method described by Lowry and Passonneau [32]. Cycling reagent (100 μl) containing 100 mmol/l Tris, 80 mmol/l HCl, 9 U/ml of type II glutamate dehydrogenase, 5 mmol/l α -ketoglutaric acid, 2 mmol/l glucose 6-phosphate,

0.1 mmol/l ADP, 10 mmol/l ammonium acetate, 0.1% (wt/vol.) BSA and 6 U/ml glucose-6-phosphate dehydrogenase was added to each tube, and tubes were then incubated for 60 min at 38°C, followed by heating to 100°C for 10 min to stop the reaction. After brief centrifugation (500 g, 2 min), a 100 μl sample was transferred to a UV cuvette that contained 0.9 ml reaction buffer consisting of 0.006 U/ml 6-phosphogluconate dehydrogenase, 30 $\mu\text{mol/l}$ NADP⁺, 0.1 mmol/l EDTA, 30 mmol/l ammonium acetate and 5 mmol/l MgCl₂ and stood at room temperature for 30 min. The NADPH formed 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 NADPH and NADP⁺ contents measured 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, oxaloacetate 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 and malate contents were measured from cell perchloric acid extract by the methods of Parvin et al. [33]. The method for malate release from isolated mitochondria has been previously described [22].

Data presentation and statistical methods All data are expressed as mean \pm SEM. The listed *n* values represent the number(s) of individual experiments 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's 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), selecting that with the strongest effects (ESM Table 1) for this study. As shown in Fig. 1a, siRNA significantly decreased PC activity (45% of control, $p < 0.01$) at 48 h after treatment. We chose 48 h of siRNA treatment because inhibition of PC activity in preliminary studies was greatest at 48 h (data not shown). A representative western blot (Fig. 1a) showed that content of the 130 kDa PC protein was reduced. Quantification of the data is shown under the protein bands. The values

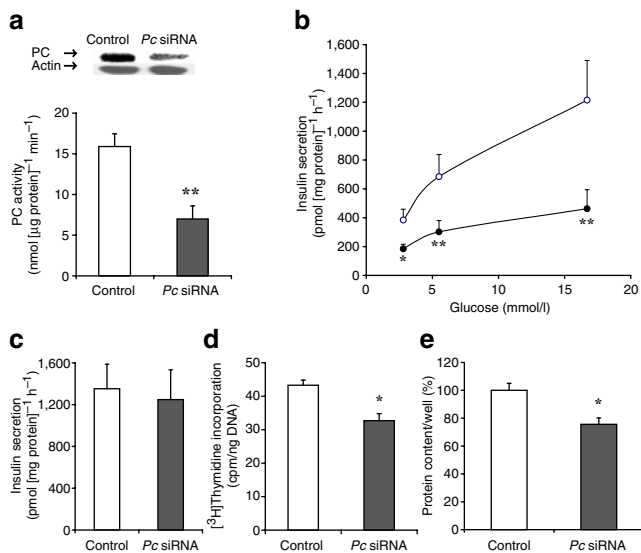
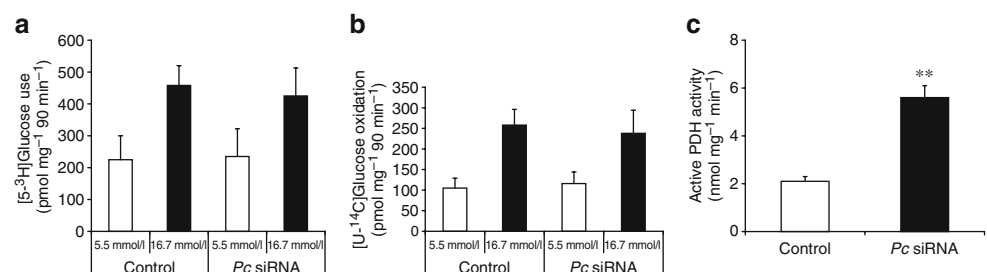


Fig. 1 *Pc* siRNA decreases PC protein content, insulin secretion, cell proliferation and protein content in INS-1 cells treated for 48 h. **(a)** PC protein content and activity. The representative western blot (of a total of three) shows PC protein (~130 kDa) and actin in control and *Pc* siRNA-treated cells. Protein band quantification (relative optical density) after normalisation to actin was 1.00 for control and 0.46 ± 0.08 ($p < 0.05$) for *Pc* siRNA. White circles, control; black circles, siRNA. **(b)** Glucose-stimulated (2.8, 5.5 and 16.7 mmol/l) insulin secretion. **(c)** KCl-stimulated (50 mmol/l) insulin secretion. **(d)** [³H]Thymidine incorporation. **(e)** Total protein content per well. Control, scrambled siRNA treatment. Data are mean \pm SEM, $n = 4$. * $p < 0.05$, ** $p < 0.01$ vs controls

of relative optical density (normalised 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 whether PC regulates insulin secretion, we inhibited PC activity by treating INS-1 cells with *Pc* siRNA for 48 h. 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 downregulated the glucose dose–response curve of insulin secretion in INS-1 cells. *Pc* siRNA, however, was unable to reduce KCl-induced insulin secretion (Fig. 1c), suggesting that the mechanism by which by KCl regulates insulin secretion is different from the PC pathway.

Fig. 2 Inhibition of INS-1 cell PC with siRNA treatment (48 h) did not change glucose use **(a)** and glucose oxidation **(b)**, but did upregulate active PDH activity **(c)** in INS-1 cells. Control, scrambled siRNA. Data are the mean \pm SEM, $n = 4$. ** $p < 0.01$



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 whether this was a causal relationship, we measured [³H]thymidine incorporation and protein content in *Pc* siRNA-treated INS-1 cells. Figure 1d demonstrates that [³H]thymidine incorporation in INS-1 cells treated for 48 h with *Pc* siRNA was decreased to 78% of control ($p < 0.05$). Parallel changes were seen in cell protein content in *Pc* siRNA-treated cells (Fig. 1e). Our data demonstrate that the specific inhibition of *Pc* expression by siRNA leads to a reduction in DNA and protein synthesis, which are measures of cell proliferation.

Inhibition of PC did not reduce glucose use 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 use and oxidation in INS-1 cells treated for 48 h with *Pc* siRNA. Surprisingly, glucose use and oxidation were not reduced (Fig. 2a, b). This result is consistent with that reported by Fransson et al. [16], who used phenylacetic acid (5 mmol/l) to inhibit PC in isolated rat islets; their results showed that phenylacetic acid was not able to inhibit glucose oxidation. We next measured PDH activity in these INS-1 cells. PDH activity was 2.7-fold increased ($p < 0.01$ vs control) (Fig. 2c). Interestingly, the increase in PDH did not compensate for the effect of reduced PC on GSIS and on beta cell proliferation (Fig. 1). The increase in PDH activity may be due to an increase in pyruvate concentration or a decrease in NADH:NAD⁺ ratio and 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 tumour cell line, we tested whether *Pc* siRNA regulates GSIS in primary beta cells. The islets isolated from male Sprague–Dawley 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 Fig. 3a, we achieved a

significant reduction in PC activity. Importantly, PC inhibition reduced GSIS significantly (Fig. 3b) when cells were stimulated with a high concentration of glucose (16.7 mmol/l). Figure 3b shows that glucose responsiveness in control group was poor. To find out the reasons for this, we tested the effect of transfection medium on primary beta cells and INS-1 cells. Our data show that GSIS in primary beta cells was significantly suppressed by the transfection medium, but INS-1 cells were less strongly affected (data not shown); this suggests chemical toxicity from the transfection medium and vulnerability in primary beta cells.

Intermediates and products of PMS and ATP content were reduced in beta cells treated with Pc siRNA Evidence that the PMS is related to insulin secretion [11, 14] prompted us to measure oxaloacetate, malate contents and NADPH:NADP⁺ ratio, since they are the intermediates or products of this shuttle. As shown in Table 1, all of these variables were significantly reduced in *Pc* siRNA-treated INS-1 cells and in dispersed islet cells. NADPH:NADP⁺ ratios were significantly increased in response to high glucose (16.7 mmol/l) in control and siRNA-treated INS-1 cells and primary beta cells. 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 1) and from dispersed islet cells (Fig. 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. For this reason we measured ATP contents in *Pc* siRNA-treated INS-1 cells. As shown in Table 1, *Pc* siRNA significantly reduced ATP contents in these cells.

Elevation of PC significantly upregulated insulin secretion and cell proliferation in stable PC overexpressing INS-1

cells Our results from *Pc* siRNA studies demonstrate that inhibition of PC reduced insulin secretion (Figs 1b, 3b) and cell proliferation (Fig. 1c, 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 twofold (Fig. 4a); insulin secretion was increased 2.5-fold at 2.8 mmol/l glucose and threefold at 16.7 mmol/l glucose (Fig. 4b). Basal insulin secretion was also slightly but significantly increased in other clones overexpressing *PC* at lower levels (data not shown), suggesting that PC also controls basal insulin secretion. The stimulatory effect of *PC* overexpression on basal insulin secretion (2.8 mmol/l glucose) (Fig. 4b) is consistent with the inhibitory effect of *Pc* siRNA on basal insulin secretion (Fig. 1b). DNA and protein synthesis was significantly increased (Fig. 4c, d). Oxaloacetate and malate contents, NADPH:NADP⁺ ratio and malate release from mitochondria were also significantly elevated in *PC* overexpressing INS-1 cells (Table 1). 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 (Fig. 2c). Despite the increase in PDH activity, GSIS was reduced (Fig. 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 PDH kinase (PDK) through phosphorylation [35]. There are four PDK isoforms in pancreatic islets [27, 36]. We reduced PDH pathway by overproducing the PDK4 isoform in INS-1 cells. The clone with the highest level of *Pdk4* overexpression was obtained and used in present study. *Pdk4* overexpression in this clone produced a great reduction in PDH activity (Fig. 5a) but no significant change in PC activity (Fig. 5b). As shown in Fig. 5c, reduced PDH

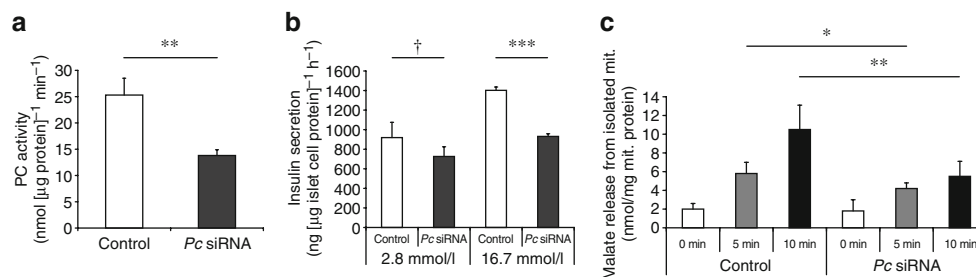


Fig. 3 *Pc* siRNA treatment (48 h) in dispersed primary islet cells reduces PC activity (a) and leads to a reduction in glucose-stimulated (16.7 mmol/l) insulin secretion (b) and malate release from isolated

mitochondria (mit.) (c). Control, scrambled siRNA. Data are the mean \pm SEM, $n=4$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; † $p=0.131$

Table 1 Metabolite contents in INS-1 cells treated for 48 h with *Pc* siRNA, in dispersed Sprague–Dawley rat islet cells and in INS-1 cells overexpressing *PC*

Cell types and variables	Control	PC	<i>N</i>	<i>p</i> values
INS-1 cells, siRNA ^a				
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 ⁺ ^c	0.35±0.05	0.26±0.07	5	0.047
NADPH:NADP ⁺ ^d	0.63±0.06 ^f	0.36±0.13	5	0.019
Malate release from mitochondria ^e	12.4±2.3	7.1±1.4	4	0.013
ATP (pmol/mg protein)	4.01±0.75	1.78±0.27	4	0.019
Dispersed rat islet cells, siRNA ^a				
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 ⁺ ^c	0.31±0.04	0.24±0.03	5	0.046
NADPH:NADP ⁺ ^d	0.58±0.15 ^f	0.32±0.12	5	<0.01
INS-1 cells, vector expression ^b				
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 ⁺ ^c	0.36±0.08	0.54±0.13	5	<0.05
NADPH:NADP ⁺ ^d	0.61±0.11 ^f	0.75±0.17 ^f	5	<0.05
Malate release from mitochondria ^e	13.4±1.7	20.6±3.2	4	0.012

Data are mean±SEM

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 h. All the cells were pre-incubated in KRB containing 0.5% BSA and different concentrations of glucose for 1 h and then the cells were washed three times with 1x pure PBS before preparation of cell extracts. Concentrations of glucose in pre-culture medium: 11.1 mmol/l (unless indicated in the table) for INS-1 cells and 5.5 mmol/l for dispersed islet cells

^a Control refers to scrambled siRNA, PC refers to *Pc* siRNA

^b Control refers to control vector, PC refers to *PC* overexpression

^c Pre-cultured with 5.5 mmol/l glucose for 1 h

^d Pre-cultured with 16.7 mmol/l glucose for 1 h

^e In nmol 10 min⁻¹ (mg mitochondrial protein)⁻¹

^f *p*<0.01 vs 5.5 mmol/l glucose

OAA, oxaloacetate

activity in INS-1 cells did not significantly change GSIS. Preliminary tests of glucose oxidation in INS-1 cells overexpressing *Pdk4* indicated that [U-¹⁴C]glucose oxidation was not significantly changed (data not shown).

Discussion

Glucose metabolism regulates insulin secretion [37–39] and blockade of 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 ones play a key role in the regulation of insulin secretion. Candidate enzymes include PC and PDH, which in pancreatic beta cells metabolise 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 vs PDH: (1) PC activity was decreased with siRNA in INS-1 cells and dispersed islet cells; (2) PC activity was increased by *PC* overexpression; and (3) PDH activity was reduced by *Pdk4* overexpression in INS-1 cells. By 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 production 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 the essential role of PC does not stem from maintenance of glucose oxidation, but rather from its ability to feed substrate into anaplerosis and the PMS.

The part of the current study involving siRNA inhibition of the *Pc* gene was also recently carried out by Jensen et al. [23]. Parts of their results are consistent with our findings, while other results differ markedly. Thus, similarly to our

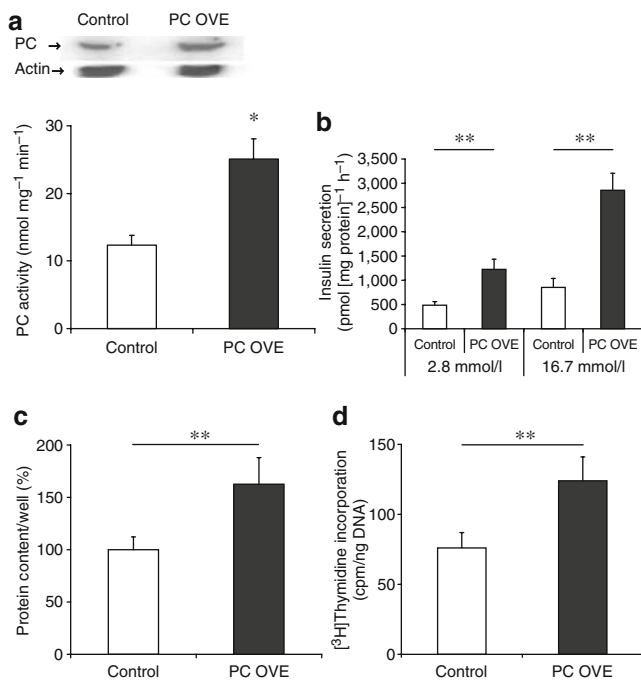


Fig. 4 Stable overproduction (OVE) of PC in INS-1 cells upregulates insulin secretion and cell proliferation. (a) PC protein content and activity, respectively. Representative western blot shows PC protein (~130 kDa) and actin (of a total of three). Protein band quantification (relative optical density) after normalisation to actin was 1.00 for control and 2.12 ± 0.12 ($p < 0.05$) for PC OVE. Glucose-stimulated (2.8 and 16.7 mmol/l) insulin secretion (b), protein content per well (c) and [³H]thymidine incorporation (d). Control cells were treated with the vector that did not contain PC gene. Data are mean \pm SEM, $n = 4$. * $p < 0.05$, ** $p < 0.01$ vs controls

data, Jensen et al. found that inhibition of PC (50% downregulation of PC) had no direct effect on either glucose use or oxidation. They also found that inhibition of PC reduced PMS, measured in our studies by malate release from mitochondria and in their studies via ¹³C NMR isotopomer analysis [23]. 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. To explain the insulin secretion result, Jensen et al. performed radioisotopic and

¹³C-NMR-based measurements of metabolic flux and mass spectrometry-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 of 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 production, suggesting a compensatory mechanism in these cells. They also believed that PC inhibition can be compensated by pyruvate–isocitrate shuttle. These are the explanations of their insulin secretion data. In our study, however, inhibition of PC depleted both malate and NADPH. We had 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 we observed large reductions in NADPH:NADP⁺ ratios and Krebs cycle intermediates in siRNA-treated INS-1 cells (Table 1), it is possible that this contributes to the reduction in DNA synthesis and protein content (Fig. 1c, d). In contrast to our results, Jensen et al. [23] found that PC inhibition had no effect on either malate content or NADPH.

We are confident of our findings, since we not only found that PC inhibition depleted NADPH and malate content, but also 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 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 in 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

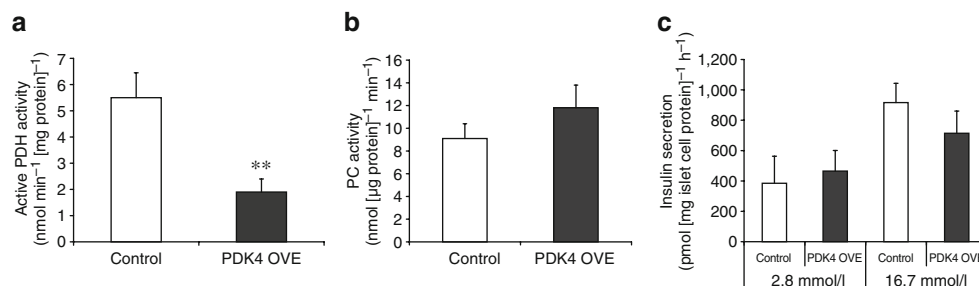


Fig. 5 Reduction of active PDH activity by overproduction (OVE) of PDK4 (a) did not significantly change PC activity (b) or glucose-stimulated (2.8 and 16.7 mmol/l) insulin secretion (c) in INS-1 cells.

Control cells were treated with the vector that did not contain the *Pdk4* gene. Data are mean \pm SEM, $n = 4$. ** $p < 0.01$

known and the reason for the different findings remains uncertain. Very recently, this group [24] provided additional evidence to support an important role of the PMS in regulation of GSIS [21]. 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 oxaloacetate, then to malate and back to pyruvate generates three important intermediates for cell proliferation: oxaloacetate, NADPH and malate. Oxaloacetate 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. Thus we reported earlier that there is a more than a twofold 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] also reported that an approximately fourfold 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 inhibit growth [46, 47], and PC activity is elevated in growing tumour cell populations [48]. The current study provides the most specific and direct evidence of PC's role in beta cell proliferation.

In summary, by manipulating PC and PDH 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, as well as 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 in the PMS pathway that makes it so important for insulin secretion.

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