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Ulrika Krus, Olga Kotova, Peter Spégel, Elna Hallgard, Vladimir V. Sharoyko, et al.. Pyruvate dehydrogenase kinase 1 controls mitochondrial metabolism and insulin secretion in INS-1 832/13 clonal β- cells. Biochemical Journal, 2010, 429 (1), pp.205-213. 10.1042/BJ20100142 . hal-00491631

HAL Id: hal-00491631
https://hal.science/hal-00491631v1

Submitted on 14 Jun 2010

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Biochemical Journal Immediate Publication. Published on 23 Apr 2010 as manuscript BJ20100142

PYRUVATE DEHYDROGENASE KINASE 1 CONTROLS MITOCHONDRIAL METABOLISM AND INSULIN SECRETION IN INS-1 832/13 CLONAL β-CELLS

Ulrika Krus[1], Olga Kotova[1], Peter Spégel[1], Elna Hallgard[1], Vladimir V. Sharoyko[1], Anna Vedin[1], Thomas Moritz[2], Mary C. Sugden[3], Thomas Koeck[1], Hindrik Mulder[1]

[1]Department of Clinical Sciences, Unit of Molecular Metabolism, Lund University Diabetes Centre, Malmö University Hospital, Sweden; [2]Umeå Plant Science Center, Swedish University of Agricultural Sciences, Umeå, Sweden; [3]Queen Mary University of London, Centre for Diabetes and Metabolic Medicine, Blizard Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, London, UK

Running title: PDH kinase 1 and insulin secretion

Address correspondence to: Olga Kotova, PhD, UMAS, Malmö CRC, entr. 72, 91:11-61, 20502 Malmö, Sweden. Tel.: +46 403-91021, Fax: +46 403-91222; E-mail: Olga.Kotova@med.lu.se

Key words: β-cell/ pyruvate dehydrogenase kinase 1/ stimulus-secretion coupling / insulin secretion / mitochondria / oxygen / electron transport / respiration / metabolism

Tight coupling between cytosolic and mitochondrial metabolism is key for glucose-stimulated insulin secretion (GSIS). Here, we examined the regulatory contribution of pyruvate dehydrogenase (PDH) kinase 1, a negative regulator of PDH, to metabolic coupling in 832/13 clonal β-cells. Knocking down PDH kinase 1 with siRNA reduced its mRNA (>80%) and protein level (>40%) after 72 h. PDH activity, glucose-stimulated cellular oxygen consumption and pyruvate-stimulated mitochondrial oxygen consumption increased 1.7- ($P<0.05$), 1.6- ($P<0.05$) and 1.6-fold ($P<0.05$), respectively. Gas chromatography/mass spectrometry revealed an altered metabolite profile upon silencing of PDH kinase 1, determined by increased levels of the TCA cycle intermediates malate, fumarate, and α-ketoglutarate. These metabolic alterations were associated with exaggerated GSIS (5-fold vs. 3.1-fold in control cells; $P<0.01$). Insulin secretion provoked by leucine and dimethylsuccinate, which feed into the TCA cycle bypassing PDH, was unaffected. The oxygen consumption and metabolic data strongly suggest that knocking down PDH kinase 1 in β-cells permits increased metabolic flux of glucose-derived carbons into the TCA cycle via PDH. Enhanced insulin secretion is likely caused by increased generation of TCA cycle-derived reducing equivalents for mitochondrial electron transport to generate ATP and/or stimulatory metabolic intermediates. Based on these findings, we suggest that PDH kinase 1 is an important regulator of PDH in clonal β-cells and that PDH kinase 1 and PDH are important for efficient metabolic coupling. Maintaining low PDH kinase 1 expression/activity, keeping PDH in a dephosphorylated and active state, may be important for β-cells to achieve the metabolic flux rates necessary for maximal GSIS.

INTRODUCTION

Type 2 Diabetes is characterized by impaired release of insulin in response to glucose. This process, or stimulus-secretion coupling, in pancreatic β-cells involves at least two pathways: the well-defined triggering pathway, i.e., a glucose-induced rise in the ATP/ADP ratio, and the less well-characterized amplifying pathway. Both pathways are dependent on efficient coupling between cytosolic and mitochondrial metabolism [1]. Here the fate of pyruvate, derived from glucose through glycolysis in the cytosol, is a key event. Pyruvate enters the mitochondrial TCA cycle either via oxidative decarboxylation to acetyl-CoA by the pyruvate dehydrogenase (PDH) complex (PDC) or via carboxylation to oxaloacetate by pyruvate carboxylase (PC; Fig. 1). Flux via lactate dehydrogenase is limited by the low activity of this enzyme in glucose-responsive pancreatic β-cells under normal oxygenated conditions [2],[3]. PC is highly expressed in pancreatic β-cells, as compared to islet non-β-cells [4], and ~ 40% of pyruvate entry into the TCA cycle during glucose stimulation occurs via its

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carboxylation by PC [5]. Hence, PC is one of the key enzymes in glucose metabolism, and its importance for glucose-stimulated insulin secretion (GSIS) has been established by us and others [6-11].

The activity of PDC is tightly regulated by substrates and products of the reaction as well as by the balance between its phosphorylation by PDH kinases and its dephosphorylation by PDH phosphatases (PDP) [12, 13]. There are four different isoforms of PDH kinases (1-4); PDH kinase 1, 2 and 4 are expressed in pancreatic islets [13-16]. The role of PDC in glucose-stimulated insulin secretion (GSIS) is more unclear than that of PC. On one hand, neither activation of PDC by dichloroacetate and adenoviral overexpression of the catalytic subunit of PDP [17], nor inactivation of PDC by overexpression of PDH kinase 3, an isoform of high activity not normally expressed in islets [18] has any effect on glucose metabolism or insulin secretion. On the other hand, it was recently shown that metabolite fluxes through PDC and PC are equally important for insulin secretion by 832/13 clonal β-cells [19]. Moreover, an increase in PDH kinase 4 protein expression in islets after fasting is associated with suppression of GSIS [20], suggesting that the kinase is important in these processes. Finally, PDC activity is significantly reduced in islets from animal models of obesity and Type 2 Diabetes [18, 21, 22].

Given the unclear but potentially important role for PDC in the control of GSIS, we decided to manipulate its activity via knock down of PDH kinase 1. This decision was also based on our finding that expression of this regulatory kinase was increased under experimental "glucotoxic" conditions in INS-1 832/13 clonal β-cells. This increased expression was associated with a blunted insulin response to glucose in 832/13 clonal β-cells (Supplementary information; Fig. S1, Table S1), a finding suggesting that PDH kinase 1 may be involved in the perturbation of GSIS in glucotoxicity. In the present studies, following knock down of PDH kinase 1 in 832/13 clonal β-cells, using small interfering RNAs (siRNA), we found that activities of PDC, the TCA cycle, and the mitochondrial electron transport system were increased concomitantly with exaggerated GSIS. This indicates that entry of glucose-derived carbons via PDC, and not only via PC, into the TCA cycle plays a regulatory role in GSIS.

EXPERIMENTAL

Reagents and siRNA

All chemicals were from Sigma (St. Louis, MO, USA) if not stated otherwise. siRNAs were 21nt long duplexes designed, synthesized and supplied by Ambion (Austin, TX, USA). Three candidate sequences against PDH kinase 1 were tested in preliminary experiments. The most efficacious sequence, used in all following experiments, was: sense strand 5'- GCAUAAAUCCAAACUGUGAtt -3' and antisense strand 5'-UCACAGUUUGGAUUUAUGCtt-3'. As negative control, Silencer® Negative Control #2 from Ambion was used.

Cell culture

INS-1 832/13 β-cells were cultured in RPMI-1640 containing 11.1 mM D-glucose and supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 µM β-mercaptoethanol, at 37º C in a humidified atmosphere containing 95% air and 5% CO_2.

Transfection

INS-1 832/13 β-cells were transfected either using Amaxa nucleofector (Amaxa, Cologne, Germany) according to the manufacturer's protocol, or by a mixture of DharmaFECT® 1 (Dharmacon; Lafayette, CO, USA) and the respective siRNA. In the Amaxa procedure, $3X10^6$ cells were resuspended in 100 µl nucleofector solution V, containing 500 nM siRNA to PDH kinase 1 or negative control, and nucleofected with program T-27. Thereafter, cells were seeded in 24- or 12-well plates and cultured for 72 hours at 11.1 mM glucose before any of the experiments were performed. When Dharmafect was employed, cells were cultured for 72 hours at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 in the presence of 35 nM siRNA (PDH kinase 1 sequence s195775) either in a XF24 V7 24-well cell culture microplates (500 µl media) or regular 12-well cell culture plates (1 ml) at 60 000 and 380 000 cells/well, respectively.

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Gene expression analysis/Q-PCR

RNA was extracted using RNAeasy (Qiagen, Hilden, Germany). 0.5 µg RNA was used for cDNA synthesis with SuperScript (Invitrogen, Carlsbad, CA, USA). 20 µl reaction mixture with 20 ng cDNA, 10 µl TaqMan mastermix (Applied Biosystems, Foster City, CA, USA), and 900 nM TaqMan gene expression assay were run in a 7900HT Fast Real-Time System (Applied Biosystems). The Q-PCR was carried out as follows: 50 ºC for 2 minutes, 95 ºC for 10 minutes, 40 cycles of 95 ºC for 15 seconds, and 60 ºC for 1 minute. The amount of mRNA was calculated relative to the amount of HPRT mRNA in the same sample by the formula $X_0/R_0 = 2^{CtR-CtX}$, where X_0 is the original amount of mRNA for the gene of interest, R_0 is the original amount of HPRT mRNA, C_tR the C_t value for HPRT, and C_tX the C_t value for the gene of interest.

Western blotting

After transfection with siRNA to PDH kinase 1 or negative control, and culture for 72 h, cells were washed with PBS and then homogenized in lysis buffer (100 mM Tris, 100 mM NaCl, 2 mM EDTA, 4.5 M urea, 1 % Triton X-100, 20 µg/ml leupeptin, 10 µg/ml antipain, and 1 µg/ml pepstatin, pH 7.4). Determination of protein concentration was performed using the BCA protein kit (Pierce, Rockford, IL). Samples were mixed with standard Laemmli loading buffer and 20 µg of protein were loaded on 10 % acrylamide gels. After SDS-PAGE proteins were electroblotted on Immun-blot PVDF membranes (BioRad). Polyclonal rabbit anti-rat PDH kinase 1 antibody (1:1500) [23] and monoclonal mouse anti-human β2A-tubulin (1:500) (Santa Cruz Biotechnology) were used as primary antibodies and horseradish peroxidase-linked goat-anti-rabbit IgG (1:6000) (Amersham Biosciences) and goat-anti-mouse IgG (1:8000) (Amersham Biosciences) were used as secondary antibodies. Blots were developed with enhanced chemiluminescence and detection was performed using Amersham Hyperfilm ECL (Amersham Biosciences). Films were scanned using the Bio-Rad GS-800 Calibrated Densitometer. For loading control blots were stripped with 2% SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.7.

Insulin secretion

Following transfection with siRNA to PDH kinase 1 or a negative control sequence, 832/13 β-cells were seeded in 24-well plates. When assayed, cells were kept in HEPES balanced salt solution (HBSS; 114 mM NaCl; 4.7 mM KCl; 1.2 mM KH_2PO_4; 1.16 mM $MgSO_4$; 20 mM HEPES; 2.5 mM $CaCl_2$; 25.5 mM $NaHCO_3$; 0.2% BSA, pH 7.2) supplemented with 2.8 mM glucose for 2 h at 37° C. Insulin secretion was then measured by static incubation of cells for 1 h in 1 ml HBSS containing 2.8 or 16.7 mM glucose or 2.8 mM glucose combined with 10 mM leucine or 10 mM dimethylsuccinate. Insulin was measured by the Coat-a-Count kit (DPC, Los Angeles, CA), which recognizes human insulin and cross reacts approximately 20% with rat insulin; INS-1 832/13 cells express human insulin [24].

Insulin content

832/13 β-cells were washed in PBS, 100 µl H_2O were added per well, and cells were scraped off and sonicated. Thereafter, cells were centrifuged and the supernatant was diluted 10X in acidified ethanol. Samples were stored at -20 ° C until assay by the Coat-a-Count kit.

PDC activity

A Dipstick assay kit for PDC activity from Mitosciences Inc. (Eugene, OR, USA) was employed. Cells transfected with siRNA to PDH kinase 1 or negative control siRNA were homogenized after 72 h by passing 5 times through the 23-G syringe needle (0.6 mm) in buffer #1, provided in the kit, and pelleted by centrifugation (10 000 x g, 5 min, $+4^0$C). 1/10 volume of detergent, provided in the kit, was added to the samples, which were then diluted to 1 mg/ml with buffer #1. Samples were added to a 96-well plate, followed by adsorption by dipsticks containing immobilized antibody to PDC, and addition of the activity buffer, which contained substrates for the PDC reaction. PDC activity was determined densitometrically from the bands with the target protein-antibody complexes formed on dipsticks, using a CCD camera (Fuji Photo Film Co. Ltd.).

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Metabolite profiling

For metabolite profiling, INS-1 832/13 clonal β-cells were transfected with siRNA to PDH kinase 1 or with a control sequence using Amaxa® as described above, and re-seeded in 6-well plates. After 72 h of culture in RPMI-1640 containing 11.1 mM glucose, efficient knock down of PDH kinase 1 was confirmed by Q-PCR as described above. Then, metabolic processes were terminated, and a cocktail of four ^{13}C-labelled internal standards, reflecting different classes of metabolites, were added to the samples. Next, polar metabolites were extracted and derivatized for separation by gas chromatography (GC) and detected by mass spectrometry (MS) as previously described with modifications [25],[26] (See Supplemental information for details). The metabolites were identified by mass spectra and retention indexes, using in-house databases as well as the National Institute of Standards and Technology (NIST) library; relative differences in individual metabolite levels were determined by calculating the resolved peak areas or areas based on specific m/z-value for each identified metabolite. Differences in the metabolite profiles between PDH kinase 1-knocked down cells and control cells were determined by multivariate analysis (See Supplemental information for details). Additionally, the metabolite levels were compared with a Student's *t*-test (See Table S2 in Supplemental information).

Oxygen consumption rate

To determine cellular and mitochondrial oxygen consumption in INS-1 832/13 clonal β-cells, a Seahorse Extracellular Flux Analyzer XF24 (Seahorse Bioscience, Billerica, USA), which measures oxygen consumption rate (OCR), was used. The instrument senses changes in oxygen content in a 7 µl volume above plated cells in XF24 V7 24-well microplates, using a fluorescence biosensor. The measurements are non-invasive and made in short and repeated intervals. For glucose-stimulated cellular oxygen consumption, an assay medium composed of 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 5.1 mM $NaHCO_3$, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2.5 mM $CaCl_2$, and 0.4% BSA (pH 7.2) supplemented with 2.8 mM glucose was used. Prior to assay, RPMI 1640 medium was removed and replaced by 750 µl of assay medium. Cells were preincubated under these conditions for 2 h at 37° in air with 5 % CO_2. The experiments were designed to determine the OCR of intact cells under low (2.8 mM) and high (16.7 mM) glucose conditions [3]. Oligomycin (OM) (5 µg/ml), which binds to and inhibits the F_O proton channel of the F_OF_1 ATP synthase, was employed to determine the OM-independent leak OCR L_i. The mitochondrial uncoupler 2,4-dinitrophenol (DNP, 40 µM) was added to determine the electron transport capacity E_i of the electron transport system (ETS). Rotenone (1 µM) was added to block transfer of electrons from complex I to ubiquinone.

The following mitochondrial function parameters were calculated: (i) the ADP stimulation/respiratory control index (RCI) calculated as the ratio between oxygen consumption at 16.7 mM glucose (coupled OXPHOS capacity P_i) and L_i at 16.7 mM glucose; (ii) the oxidative phosphorylation (OXPHOS) limitation ratio that is controlled by the phosphorylation system and calculated as the ratio between P_i and E_i; and (iii) electron transport coupling ratio (ECR) calculated as the ratio between L_i and E_i.

For pyruvate-stimulated mitochondrial oxygen consumption, assay media A (114 mM NaCl, 4.7 mM KCl, 1.16 mM $MgSO_4$, 1.2 mM KH_2PO_4, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5.1 mM $NaHCO_3$, 16.7 mM glucose, pH 7.2) and B (114 mM NaCl, 4.7 mM KCl, 5.5 mM KH_2PO_4, 5.1 mM $NaHCO_3$, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1.16 mM $MgSO_4$, 0.54 mM EDTA, 0.04 % BSA, pH 7.2) were prepared. Prior to assay, RPMI 1640 medium was removed and replaced by 750 µl of assay medium A. Cells were preincubated under these conditions for 20 min at 37° in air with 5% CO_2. Then assay medium A was removed and replaced with 50 µl of assay medium B with 10 µg/ml digitonin to permeabilize the cells. After 5 min at 37° in air with 5% CO_2, 700 µl of assay medium B were added. The experiments with permeabilized cells were designed to directly determine mitochondrial oxygen consumption with 7.5 mM pyruvate as substrate in the presence (P_m, state 3) and absence (L_m, state 4) of 400 µM ADP. DNP (40 µM) was added to determine E_m, the oxygen consumption of uncoupled mitochondria in the presence of excess metabolic substrate. Then, 5 µM rotenone were added to block transfer of electrons from complex I to ubiquinone. Tests with cytochrome C addition during OCR measurements confirmed that digitonin did not cause mitochondrial damage. Mitochondrial function parameters used were: (i) ADP stimulation

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or RCI calculated as P_m divided by L_m; (ii) OXPHOS limitation ratio calculated as P_m divided by E_m; and (iii) ECR calculated as L_m divided by E_m.

Statistical analysis
All data are presented as mean ± S.E.M. Statistical analysis was performed using paired and unpaired Student's *t*-test. PDC activity data, which were arbitrary, were analyzed by a non-parametric Wilcoxon signed rank test. A probability level less than 0.05 was considered significant.

RESULTS

Knock down of PDH kinase 1
Fig. 1 shows the main metabolic pathways through and associated with the TCA cycle and the key regulatory steps. PDH kinases phosphorylate the PDC, rendering it inactive, and thereby control the flux of carbons from pyruvate into the TCA cycle. Expression of PDH kinase 1 was selectively increased after culture of INS-1 832/13 clonal β-cells at 16.7 mM glucose for 48 h (>2-fold increase in PDH kinase 1 mRNA compared with a 0.5-fold increase in PDH kinase 2 mRNA; Supplemental data, Fig. S2). As a result of the exposure to hyperglycaemia, insulin secretion was impaired (Supplemental data, Fig. S1). Thus, we hypothesized that enhanced expression of PDH kinase 1 might be involved in the aetiology of the impaired GSIS that occurs in response to long-term exposure of the β-cells to high glucose concentrations. This suggests that PDH kinase 1 is an important regulator of β-cell metabolism and GSIS. To address this possibility, we decided to investigate the effect of silencing PDH kinase 1 on metabolism, mitochondrial function, and insulin secretion in clonal pancreatic β-cells. To this end, INS-1 832/13 clonal β-cells were treated with siRNA against PDH kinase 1. This led to a decrease in the PDH kinase 1 mRNA level by >80%, as quantified by Q-PCR (Fig. 2A); accordingly, PDH kinase 1 protein expression was decreased as well (by >40%), as shown by Western blot (Fig. 2B).

PDC activity after knock down of PDH kinase 1
To confirm that knock down of PDH kinase 1 would have the expected metabolic impact on its down stream target, we performed measurements of PDC enzyme activity. Indeed, PDC activity was significantly higher in 832/13 clonal β-cells with knocked down PDH kinase 1 compared to negative control (1.7-fold increase; $P<0.05$) (Fig. 2C).

Insulin secretion and content after knock down of PDH kinase 1
Next, we examined the effect of knock down of PDH kinase 1 on insulin secretion in response to glucose and two additional nutrient secretagogues, leucine and dimethylsuccinate (Fig. 3). INS-1 832/13 clonal β-cells nucleofected with the negative control increased insulin secretion by 3.1-fold over basal (2.8 mM glucose) when stimulated with 16.7 mM glucose; this fold-response is lower than what we normally observe in untreated INS-1 832/13 clonal β-cells (Supplemental information, Fig. S1), and can most likely be attributed to the nucleofection procedure. Knock down of PDH kinase 1 did not affect basal insulin secretion but, remarkably, insulin secretion in response to 16.7 mM glucose increased more vigorously than in negative control cells (5-fold; $P<0.01$ versus negative control cells). In contrast, insulin secretion provoked by the two other secretagogues, leucine and dimethylsuccinate (Fig. 3), which are metabolized in mitochondria but without the requirement of PDH, was not influenced by knock down of PDH kinase 1. In response to leucine, cells with knocked down PDH kinase 1 increased insulin secretion 4.7-fold over basal secretion at 2.8 mM glucose, while negative control cells responded 3.9-fold. In response to dimethylsuccinate, cells with knocked down PDH kinase 1 increased insulin secretion 3.8-fold versus basal (2.8 mM glucose); negative control cells responded 3.3-fold. There was no significant change in insulin content of 832/13 clonal β-cells when PDH kinase 1 had been knocked down compared to negative control cells (616.6 ± 214.1 vs 470.7 ±192.7 ng insulin/mg protein; n=5).

Metabolite profiling in 832/13 clonal β-cells We hypothesized that knock down of PDH kinase 1 would relieve inhibition of PDC, leading to increased decarboxylation of pyruvate to acetyl-CoA and, potentially, increased funnelling of glucose-derived carbons into the TCA cycle. This would

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potentially change the levels of the TCA cycle intermediates and/or alter the flux through the TCA cycle. Exit of metabolites from the TCA cycle (cataplerosis) could also serve as coupling signals in the amplifying pathway of insulin secretion. To address the former possibility, we undertook a comprehensive profiling of polar metabolites extracted from 832/13 clonal β-cells, using GC/MS. In this approach, 380 resolved components (corresponding to putative derivatized metabolites) could be distinguished and relatively quantified 72 h after treatment of cells with siRNA to PDH kinase 1 or a control sequence. To this end, we identified 80 out of the 380 putative metabolites, comprising most glycolytic and TCA cycle intermediates, several amino acids and a number of fatty acids (Supplemental data; Table S2). Employing multivariate analysis, i.e., principal component analysis (PCA) and orthogonal partial least squares discriminate analysis (OPLS-DA) [27], we found that the metabolite profile of cells where PDH kinase 1 had been knocked down was significantly different from that of the control cells (Fig. 4A). Interestingly, we found that the TCA cycle intermediates malate, fumarate, and α-ketoglutarate were the strongest determinants of the clustering in the statistical model and, hence, the differential nature of the metabolite profile upon the knock down of PDH kinase 1 (Fig 4B; Supplemental data; Table S2). This could be interpreted as a reflection of increased activity in the TCA cycle and, possibly also in mitochondrial metabolite shuttles. Importantly, malate is an important component of cycles from citrate or oxaloacetate to pyruvate, and is required for generation of NADPH when malate is oxidized to pyruvate by malic enzyme. Also the level of glycerol-3-phosphate was significantly increased. This metabolite is part of the glycerolphosphate shuttle, which is required for the exchange of reducing equivalents between glycolysis and the TCA cycle, and which has been shown to play an important role in β-cell stimulus-secretion coupling [28]. Interestingly, the level of β-hydroxybutyrate, a ketone body, was reduced in cells with knocked down PDH kinase 1. This could be interpreted as increased formation of citrate from the condensation of acetyl-CoA (increasingly provided by PDC-catalyzed formation from pyruvate) and oxaloacetate, thereby depleting acetyl-CoA as a precursor of β-hydroxybutyrate.

Oxygen consumption

Having established that activity in the TCA cycle was increased, as evident from increases in levels of critical intermediates, we asked whether such increases would affect OXPHOS and therefore ATP synthesis. The observed glucose-stimulated increase in TCA cycle activity should generate more of the reducing equivalents NADH and $FADH_2$, donating electrons to the mitochondrial ETS. This in turn should increase the reduction of O_2 by cytochrome C oxidase (complex IV) and generate the electrochemical potential necessary to boost OXPHOS. Thus, as complex IV activity is responsible for more than 90% of total cellular oxygen consumption [29], measurements of cellular and even more so mitochondrial OCRs were used to examine metabolite-stimulated alterations of mitochondrial OXPHOS. Indirectly, these measurements reflected metabolic activity of mitochondria. In pancreatic β-cells, this is highly relevant because rises in the ATP/ADP ratio and metabolic intermediate levels control insulin secretion, i.e., stimulus-secretion coupling.

OCR measurements by the Seahorse XF24 showed that intact glucose-responsive INS-1 832/13 clonal β-cells progressively and significantly increased their OCR by an average of 2.33 ± 0.13 nmol O_2 / min / mg protein in response to 16.7 mM glucose (P_i) compared to the basal OCR at 2.8 mM glucose (Fig. 5A). When PDH kinase 1 was knocked down in the 832/13 β-cells, the glucose-induced increase in the OCR was significantly exaggerated, reaching 3.08 ± 0.30 nmol O_2 / min / mg protein ($P<0.05$). This effect of PDH kinase 1 knock down coincided with: (i) an increased ADP-stimulation driven by the proton gradient dissipation through the ATP synthase. This was calculated as the difference between P_i and L_i (3.66 ± 0.20 in control cells vs. 4.27 ± 0.19 nmol O_2/ min / mg protein in PDH kinase 1 knock down cells; $P<0.05$) and (ii) an increased L_i, and E_i (Fig. 5A). In contrast, the functional indicators RCI, ECR and OXPHOS limitation ratio showed no significant change (Supplemental data; Table S3).

Given that PDH kinase 1 knock down altered pyruvate metabolism, we next directly measured pyruvate-stimulated mitochondrial oxygen consumption in digitonin-permeabilized 832/13 β-cells. We found that in comparison to control cells, PDH kinase 1 knock down increased the P_m (state 3), L_m, (state 4), and E_m (DNP) as well as the rotenone-independent OCR (Fig. 5B). As in intact cells, PDH kinase 1 knock down did not change RCI, OXPHOS limitation ratio, and ECR (Supplemental data;

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Table S3).

The exaggeration of the coupled OXPHOS capacity in intact and permeabilized 832/13 β-cells was expected, and confirmed that there was an increased availability of reducing equivalents upon silencing of PDH kinase 1. More so, the metabolomic data on the abundance of malate, α-ketoglutarate, and fumarate, together with the observed proportionally similar general increase of coupled OXPHOS capacity and electron transport capacity of the ETS indicate that PDH kinase 1 knock down leads to increased metabolic and OXPHOS fluxes in mitochondria in response to glucose stimulation. This is likely to increase ATP generation. The data further indicate an increased potentially controlled/induced and functionally important proton conductance under non-stimulatory conditions after PDH kinase 1 knock down as the leak OCR increase was also proportionally similar while the ECR was unaltered.

The observed large, but PDH kinase 1 knock down-independent excess capacity of the ETS in intact cells as well as mitochondria of permeabilized cells, expressed as the OXPHOS limitation rate, indicated a pronounced PDC-independent limitation of the OXPHOS capacity by the phosphorylation system (ATP synthase, ANT, phosphate transporter). Thus, we conclude that in INS-1 832/13 clonal β-cells, the metabolic flux from the cytosol into mitochondria, at least through PDC, has no substantial influence on OXPHOS limitation. Nonetheless, it will be necessary to further investigate this limitation as it may potentially alter stimulus-secretion coupling through the sensitive phosphorylation state of the ATP pool in the cytoplasm.

To confirm the OXPHOS-dependent increase in ATP generation, we have tried to determine differences in ATP/ADP ratios in our experimental model using conventional biochemical techniques [7]. While an elevation of glucose from 2.8 to 16.7 mM robustly increased the ATP/ADP ratio (primarily via reduction of ADP; data not shown), we failed to identify an exaggeration of the ATP/ADP ratio in INS-1 832/13 β-cells upon knock down of PDH kinase 1. This could be explained by the complications arising from a potentially prevailing compartmentalized increase that would not be detected in a cellular extract. Another possibility is timing; we analyzed accumulation of the nucleotides at 5 and 30 minutes after elevation of glucose – perhaps at another time point, a differential rise in the ATP/ADP ratio will be apparent.

Overall, the oxygen consumption results indicate that PDH kinase 1 knock down was not associated with functional alterations in the mitochondrial OXPHOS system *per se*. Instead, they further substantiated our initial conclusion that PDH kinase 1 knock down leads primarily to increased metabolic flux and therefore enhanced metabolite-stimulated OXPHOS. The consequence of this will be more metabolically generated ATP.

DISCUSSION

The present studies are an extension of the efforts of a number of laboratories to resolve in which way fuel metabolism controls insulin secretion from the pancreatic β-cell. On a comprehensive level our studies reinforce the concept that the precise way in which glucose-derived carbons are metabolized in the mitochondrion plays a more important role in GSIS than the overall rate of cellular glucose metabolism.

While the majority of the recent studies concerning stimulus-secretion coupling has addressed the role of carbons entering into the mitochondrial TCA cycle via the PC-catalyzed reaction [7, 9, 10, 19], we have examined the role of the PDC. This initiative was prompted both by the well-known importance of PDC for the control of TCA cycle activity, and by our finding that PDH kinase 1 was upregulated in 832/13 clonal β-cells exposed to high glucose resulting in impaired GSIS (Supplemental information, Fig. S1-2). It raised the possibility that PDH kinase 1 is part of the metabolic control of GSIS. PDH kinase 1 is one of four isoforms of a kinase, inactivating the PDC via phosphorylation of three serine-residues on PDC E1α: serine-264 (site 1), serine-271 (site 2) and serine-203 (site 3) [13, 30]. All PDKs can phosphorylate site 1 and site 2, whereas PDH kinase 1 uniquely phosphorylates site 3 [31]. This suggests that site 3 is of special importance in tissues where PDH kinase 1 is expressed. Indeed, PDH kinase 1 is expressed in pancreatic islets [20] and clonal β-cells (shown here), heart [32] and, to a lesser extent, skeletal muscle [33]. Since reactivation of E1α requires removal of all three phosphate groups, PDH kinase 1 phosphorylation of site 3 could be particularly effective in maintaining PDC inactive.

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Previous studies have reported discrepant data regarding the potential roles of the PDH kinases in modulating insulin secretion [17]. While our examination shows a clear effect on GSIS and metabolism, such effects were lacking in a previous study, which employed adenoviral overexpression of PDH kinase 3 [17], an isoform not expressed in 832/13 clonal β-cells. The discrepancy may be attributed to the differential affinity of the PDH kinases for phosphorylation sites on PDC E1α. Interestingly, while the activity of the PDH kinases is inhibited by pyruvate, that of PDH kinase 1 is the least susceptible to inhibition by this critical metabolite [23]. Thus, PDH kinase 1 would be predicted to be less inactivated compared to other PDH kinase isoforms by an acceleration of glycolytic flux. The regulation of PDH kinase 1 would thus be analogous to that of glucokinase versus hexokinase, where the former lacks product inhibition by glucose-6-phosphate, rendering it more suitable for the role as "glucosensor" in pancreatic β-cells and hepatocytes.

On a more detailed mechanistic level, knocking down PDH kinase 1 provided novel insight into the metabolic coupling of stimulus to secretion in clonal β-cells. Setting the stage for our studies, knock down of PDH kinase 1 decreased both PDH kinase 1 mRNA and protein levels, and promoted a significant enhancement of GSIS. Moreover, an increase in PDC activity was observed, indicating that decreased PDH kinase 1 expression is translated to decreased PDC phosphorylation and, consequently, increased PDC activity. A PDC activity-dependent increase in the import of glucose-derived carbons, i.e. from pyruvate, into mitochondria and the associated enhancement of metabolic flux within mitochondria, i.e. the TCA cycle, are in good agreement with the PDH kinase 1 knock down-associated increases in levels of the TCA cycle intermediates malate, fumarate, and α-ketoglutarate. Such increases also require anaplerotic input of carbons into the TCA cycle, producing more oxaloacetate, which can be condensed with acetyl-CoA to form citrate and the subsequent TCA cycle intermediates. Anaplerotic activity in clonal β-cells is now well-established [7, 9, 10, 19]. The mechanism is further supported by our observation, that insulin secretion stimulated by leucine or dimethylsuccinate, which enter the TCA cycle independent of PDH activity, was unaffected by knock down of PDH kinase 1. Furthermore, glucose-stimulated increases in citrate, malate and α-ketoglutarate have previously been described in INS-1 cells, using biochemical analyses [6, 34]. We also know from previous studies [2, 3] that competing metabolic fluxes like lactate formation are unlikely, since lactate dehydrogenase activity is very low in the 832/13 β-cells [3].

In the attempt to elucidate the mechanism behind the observed enhancement of GSIS in 832/13 clonal β-cells upon knocking down PDH kinase 1, we hypothesized that increased PDH activity would tighten the metabolic coupling between the cytosol and mitochondria; more pyruvate would be decarboxylated, forming acetyl-CoA for subsequent generation of citrate from oxaloacetate. Tightened metabolic coupling should hence increase TCA cycle metabolism and thus the metabolite flux-controlled mitochondrial synthesis of ATP via OXPHOS. Pyruvate cycling, which requires the action of PC, and is positively correlated with glucose responsiveness in 832/13 clonal β-cells, would be a complementing metabolic flux [9]. The exact path of this cycle was not determined, and it assumed to exert its effects via metabolic intermediates, e.g., NADPH [35],[36], acting downstream on exocytotic control elements [9],[37].

Glucose stimulation of insulin secretion via an increased ATP/ADP ratio based on metabolite-stimulated OXPHOS necessitates elevated mitochondrial oxygen consumption. The proton circuit across the inner mitochondrial membrane links the reducing equivalent-dependent electron transport, oxygen consumption, and ATP generation, i.e., OXPHOS. Indeed, PDH kinase 1 knock down caused a pronounced metabolite stimulation of cellular oxygen consumption. Thus, our present studies clearly indicate that in glucose-responsive 832/13 clonal β-cells, PDH kinase 1-regulated PDC activity contributes to β-cell stimulus-secretion coupling via a metabolite-driven increase in OXPHOS-based ATP production. Moreover, we could rule out any PDH kinase 1 knock down-dependent fundamental functional alterations in the OXPHOS system besides electron transport maximization in the ETS. Our data further indicate that the metabolite stimulation was based on an increased metabolic flux of cytosolic glucose-derived carbons into mitochondria via PDC, in turn increasing metabolic flux through the TCA cycle. This increased metabolic flux apparently increased the generation of NADH and $FADH_2$, which, in turn, stimulated the activity of the mitochondrial ETS and therefore oxygen consumption. However, when ADP levels fall and the ATP/ADP ratio approaches an equilibrium, excess carbons may leave the TCA cycle (cataplerosis), particularly in a cell such as the β-cell, where PC activity is known to be high [5], driving anaplerosis [9]. Such exit of metabolic intermediates has

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been suggested to be part of β-cell stimulus-secretion coupling [36], and is also suggested by our findings of increased levels of α-ketoglutarate, fumarate, and malate.

A reduced level of β-hydroxybutyrate, as observed upon PDH kinase 1 knock down, would be expected to occur if β-oxidation is restricted or if acetyl-CoA levels drop as a consequence of increased formation of oxaloacetate. Indeed, citrate exiting from mitochondria would lead to cytosolic production of acetyl-CoA by the reaction catalyzed by citrate lyase, which in turn may be carboxylated to malonyl-CoA. Malonyl-CoA is a strong allosteric inhibitor of carnitine palmitoyl transferase 1, thus preventing flux of long-chain acyl-CoAs into the mitochondrion for subsequent β-oxidation. This presumed elevation of cytosolic long chain acyl-CoAs could potentially have been stimulating exocytosis [38, 39] and/or influenced proton conductance. However, the support for the role of malonyl-CoA in stimulus-secretion coupling in 832/13 clonal β-cells has not been unambiguous [40, 41]. Whether malonyl-CoA production is enhanced upon silencing of PDH kinase 1 is not known, but silencing of acetyl-CoA carboxylase 1 in 832/13 clonal β-cells and rat islets has recently been reported to suppress GSIS via inhibition of glucose rather than lipid metabolism [42]. The possibility that acetyl-CoA levels drop, indicated by reduced β-hydroxybutyrate levels, agrees with increased anaplerosis, a phenomenon that has been repeatedly shown in clonal β-cells [7, 9, 10, 19]. Anaplerosis was further indicated by the fact that levels of α-ketoglutarate, fumarate, and malate rose upon silencing of PDH kinase 1.

In conclusion, we have identified a potential role for PDH kinase 1 in the regulation of glucose responsiveness in clonal β-cells. The mechanisms of this role are illustrated by a cartoon (Fig. 6). We suggest that maintaining low PDH kinase 1 expression, keeping PDC in a relatively dephosphorylated and active state, plays an important role for robust GSIS in clonal β-cells. In the event that the role of PDH kinase 1 in GSIS can be confirmed as a more general mechanism for metabolic control of insulin secretion, it may prove to be a target for glucose-dependent insulinotropic drugs in treatment of failing insulin secretion in Type 2 Diabetes.

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ACKNOWLEDGEMENTS

We thank Professor R.A. Harris (Indianapolis, U.S.A.) for generously providing us with anti-PDH kinase 1 antibodies, generated in rabbits against recombinant PDH kinase 1 protein. These studies were supported by the Swedish Research Council [14196-06-3], the Crafoord and Albert Påhlsson Foundations, the Swedish Diabetes Association, Diabetes UK [DMMG1D7R and DMMG1C5R] and the Faculty of Medicine at Lund University.

Abbreviations used: ACC, acetyl-CoA carboxylase; DNP, 2,4-dinitrophenol; ECR, electron transport coupling ratio; ETS, electron transport system; Fum, fumarate; GC/MS, gas chromatography/mass spectrometry; Gly 3P, glycerol-3-phosphate; HBSS, HEPES balanced salt solution; HPRT, hypoxanthine-guanine phosphoribosyl transferase; β-HB, β-hydroxybutyrate; K_{ATP}-channels, ATP-sensitive K+ channels; α-KG, α-Ketoglutarate; LDA, low density array; Mal, malate; MS, mass spectrum; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PC, pyruvate carboxylase; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase, Q-PCR, quantitative PCR; RCI, respiratory control index; siRNA, small interfering RNA; TCA cycle, tricarboxylic acid cycle.

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FIGURE LEGENDS

Fig.1. Metabolic pathways in the mitochondrion in pancreatic β-cells.

Fig. 2. Knock down of PDH kinase 1 (white bares represent siNC, black ones siPDK1). (A) PDH kinase 1 mRNA levels relative to HPRT in negative control and siRNA-treated cells 72 h after transfection. Values are means ± S.E.M. for 4 independent experiments; *** P<0.001. (B) Western Blot analyses of PDH kinase 1 protein expression in negative control and siRNA-treated cells 72 h after transfection. PDK1 and β2A-tubulin blots are shown and values are means ± S.E.M. for 4 independent experiments; ** P<0.01 in unpaired t-test and Wilcoxon signed rank test. (C) PDC activity is increased by silencing of PDH kinase 1. 832/13 INS-1 cells transfected with siRNA to PDH kinase 1 and negative control were cultured for 72h. Dipstick assay was used to measure PDC activity; quantification of PDC activity is shown. Values are means ± S.E.M. for 4 independent experiments. * P<0.05 in Wilcoxon signed rank test (arbitrary values were compared) and paired t-test.

Fig. 3. Glucose-stimulated insulin secretion is enhanced by knock down of PDH kinase 1. 72h after siRNA transfection, 832/13 clonal β-cells were stimulated with 2.8 mM glucose, 16.7 mM glucose or 2.8 mM glucose combined with 10 mM leucine or 10 mM dimethylsuccinate, respectively, during 1h. Values are means ± S.E.M. for 15 independent experiments for GSIS and 8 independent experiments for leucine and dimethylsuccinate (white bares represent siNC, black ones siPDK1). ** P<0.01.

Fig.4. Metabolite profiling of 832/13 clonal β-cells. (A) Metabolites were extracted and derivatized from thirty 6-well plates of 832/13 clonal β-cells subjected to silencing of PDH kinase 1 (72 h) or control cells. Metabolites were profiled by GC/MS. Metabolites under the two different conditions form 2 clusters, indicating that the total metabolite profiles are significantly different; the analysis was performed by the supervised method OPLS-DA (See Supplemental data for details). (B) The relative abundance of selected TCA cycle intermediates is illustrated in a bar graph (white bares represent siNC, black ones siPDK1). The levels in control cells are set to 100%; comparisons were made by Student's *t*-test. * P<0.05; ** P<0.01. α-KG, α-Ketoglutarate; Fum, fumarate; Mal, malate; β-HB, β-hydroxybutyrate; Gly 3P, glycerol-3-phosphate.

Fig.5. Analysis of oxygen consumption rates (OCR) upon PDH kinase 1-silencing in 832/13 clonal β-cells, using Seahorse XF 24 flux analyzer. (A) Cells were preincubated at 2.8 mM glucose for 2 h. Then OCRs were measured first at 2.8 mM followed by measurements at 16.7 mM after glucose injection at time point 1. At time point 2., oligomycin, an inhibitor of ATP synthase was added; at time point 3, dinitrophenol, an uncoupler was added, followed by rotenone at time point 4, an inhibitor of complex 1 (white squares represent siNC, black ones siPDK1). (B) Next, 832/13 clonal β-cells were permeabilized and exposed to pyruvate. OCR for P_m (state 3, excess ADP) and L_m (state 4, ADP consumed) as well as for DNP-uncoupled mitochondria in the absence (E_m) and presence of rotenone (E_m / rotenone) are given (white bares represent siNC, black ones siPDK1). Comparisons were made by paired Student's *t*-test due to differences between passages. * P<0.05; ** P<0.01.

Fig.6. Cartoon illustrating the metabolic events upon silencing of PDH kinase 1 underlying enhanced glucose-stimulated insulin secretion. Direction of arrows indicates whether a reaction or level is increased or decreased; italics indicate that the event is inferred but not shown by our studies. Pyruvate dehydrogenase (PDH), Pyruvate dehydrogenase complex (PDC), Acetyl-CoA_m – mitochondrial Acetyl-CoA, Tricarboxylic acid (TCA), electron transfer (ET), oxidative phosphorylation (OXPHOS), glucose-stimulated insulin secretion (GSIS).

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Figure 1

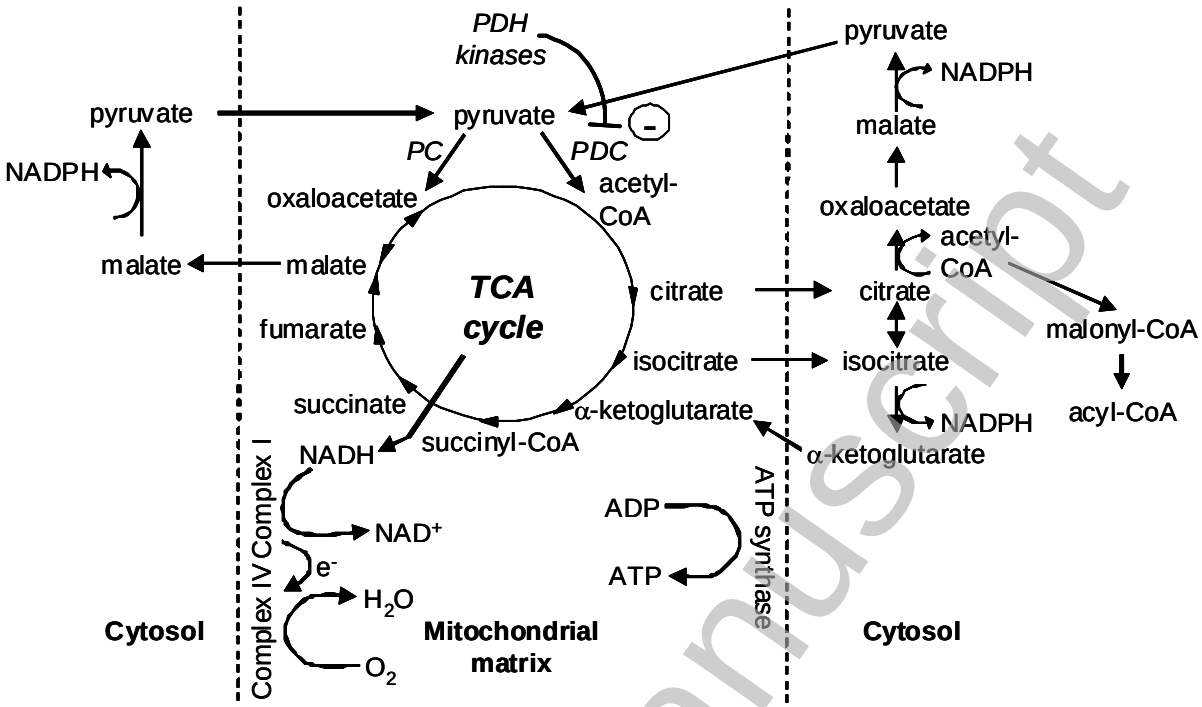

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Figure 2

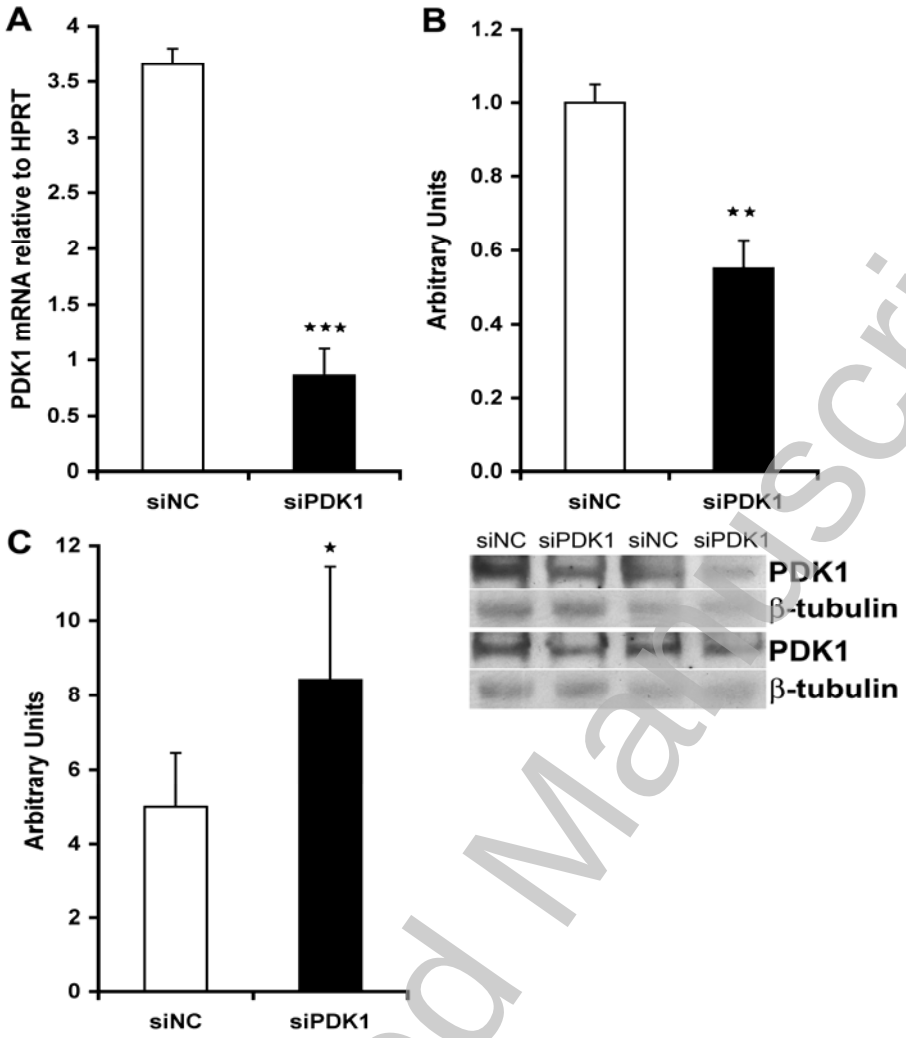

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Figure 3

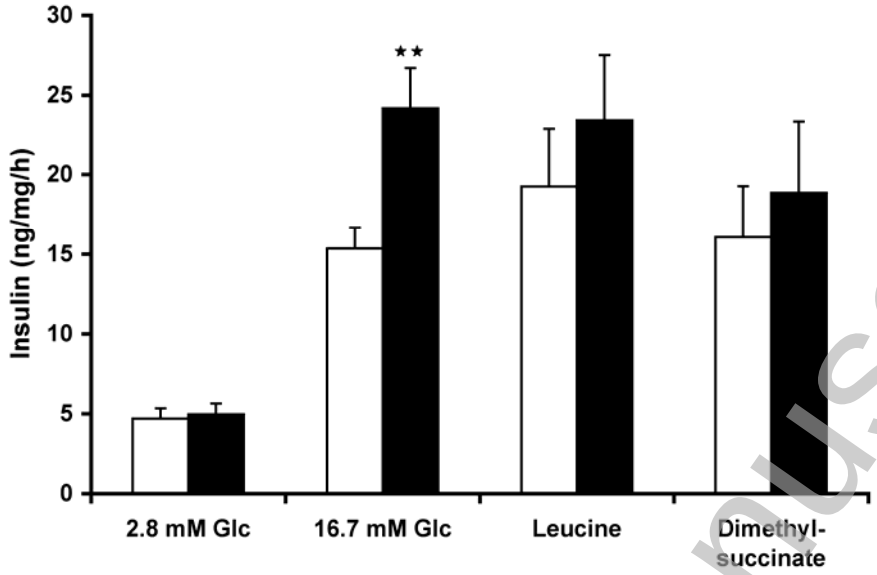

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Figure 4

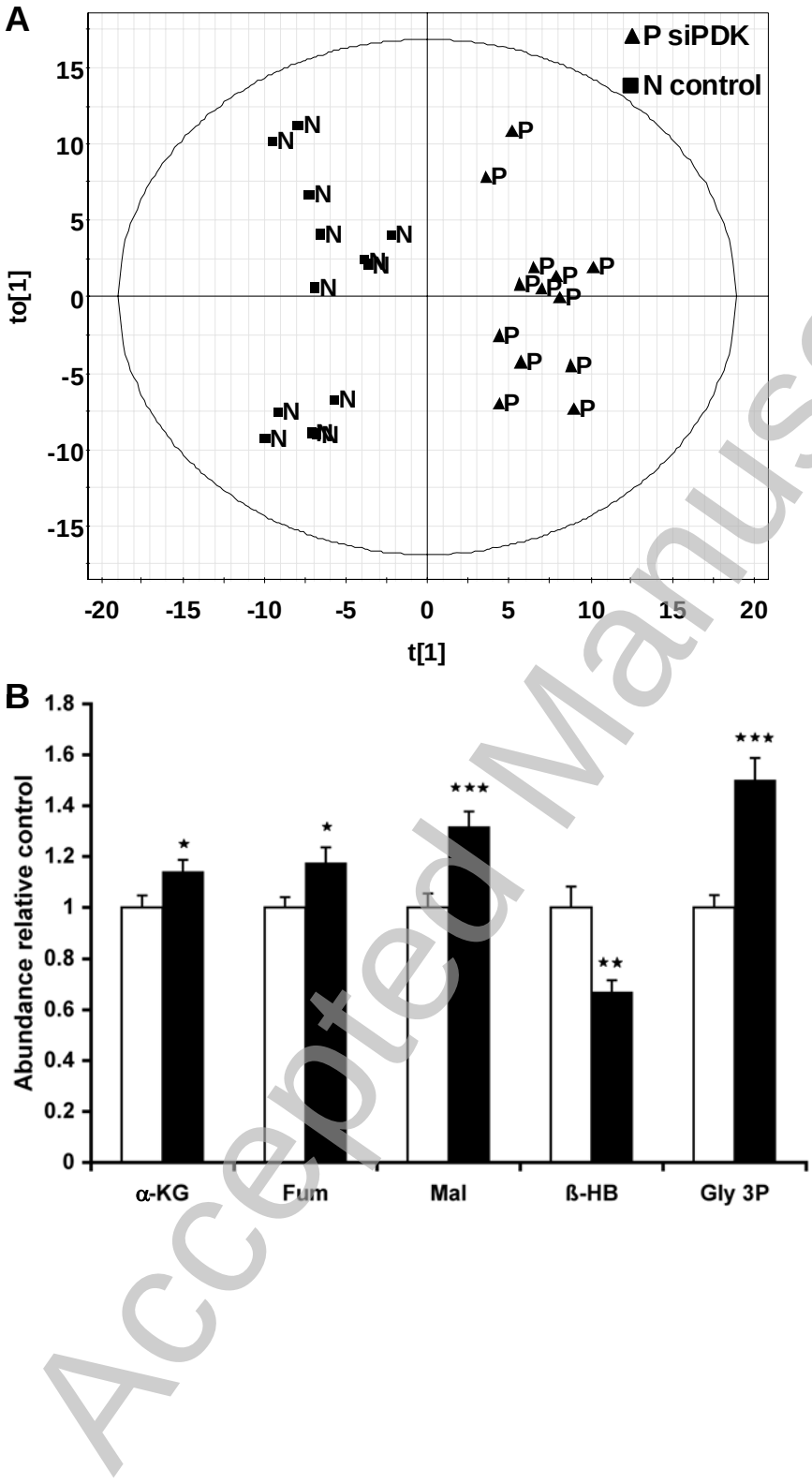

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Figure 5

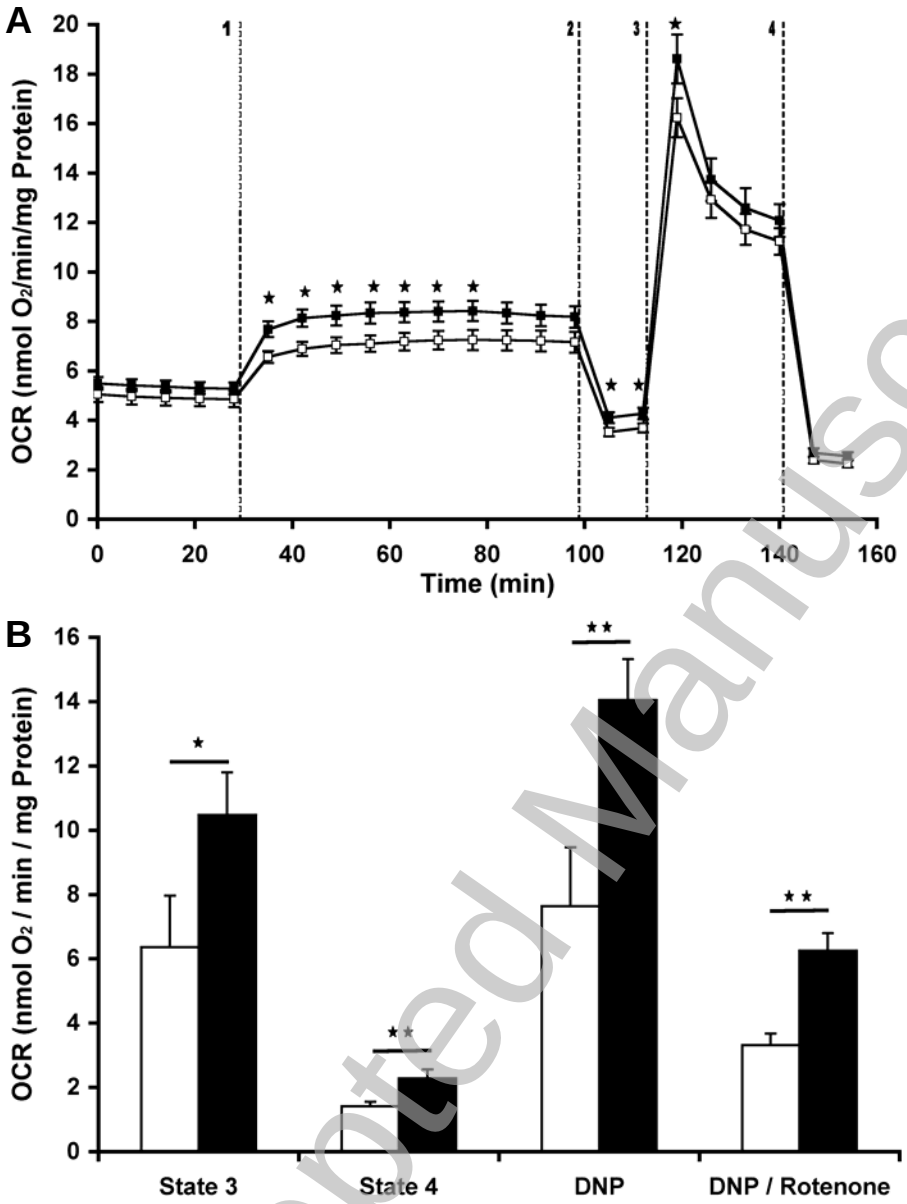

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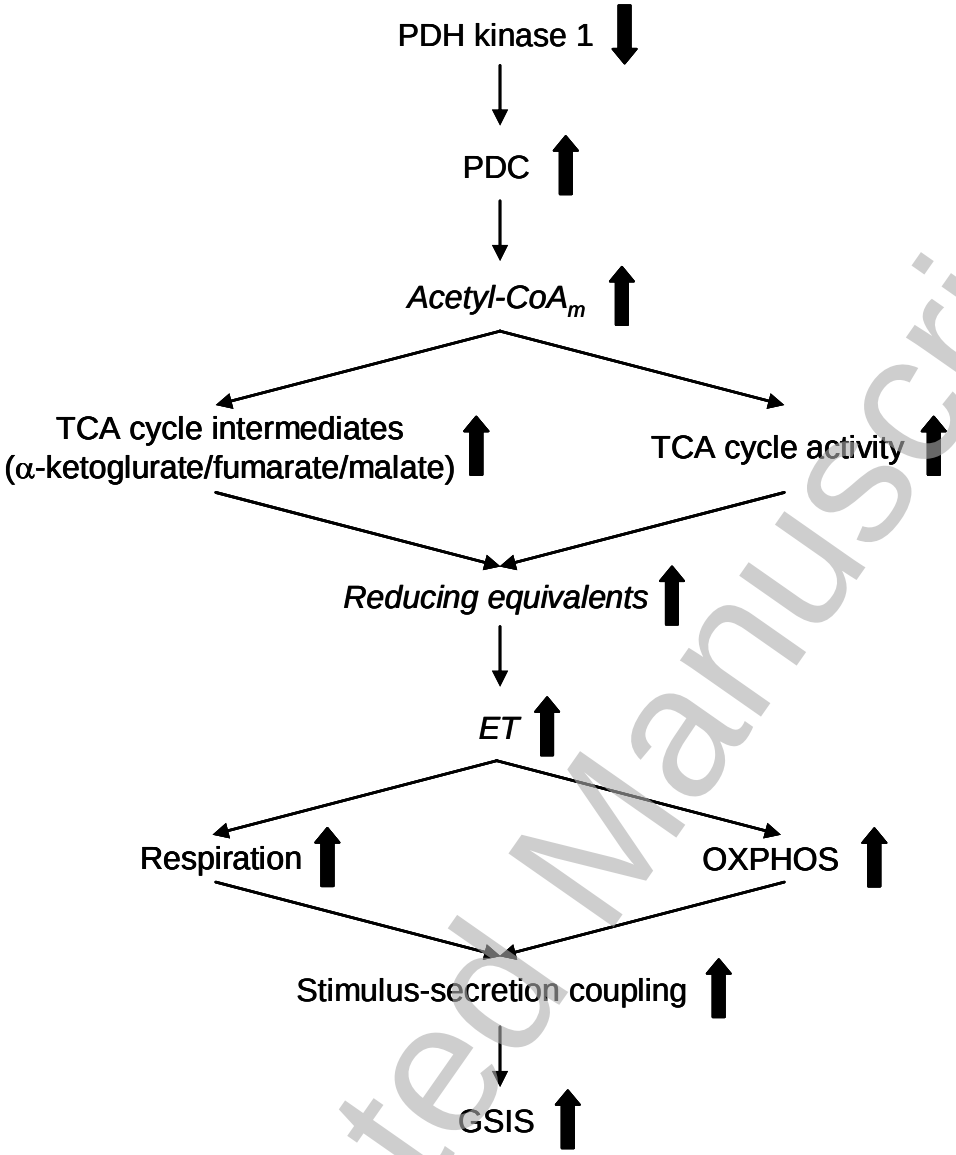
Figure 6



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