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Abstract
Glucose-induced insulin secretion from pancreatic β-cells depends on mitochondrial activation. In the organelle, glucose-derived pyruvate is metabolised along the oxidative and anaplerotic pathway to generate downstream signals leading to insulin granule exocytosis. Entry into the oxidative pathway is catalysed by pyruvate dehydrogenase (PDH) and controlled in part by phosphorylation of the PDH E1α subunit blocking enzyme activity. We find that glucose but not other nutrient secretagogues induce PDH E1α phosphorylation in INS-1E cells and rat islets. INS-1E cells and primary β-cells express pyruvate dehydrogenase kinase (PDK) 1, 2 and 3, which mediate the observed phosphorylation. In INS-1E cells, suppression of the two main isoforms, PDK1 and PDK3, almost completely prevented PDH E1α phosphorylation. Under basal glucose conditions, phosphorylation was barely detectable and therefore the enzyme almost fully active (90% of maximal). During glucose stimulation, PDH is only partially inhibited (to 78% of maximal). Preventing PDH phosphorylation in situ after suppression of PDK1, 2 and 3 neither enhanced pyruvate [...]

Reference

DOI : 10.1016/j.bbamcr.2012.07.005
PMID : 22809973
Pyruvate dehydrogenase E1α phosphorylation is induced by glucose but does not control metabolism-secretion coupling in INS-1E clonal β-cells

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ABSTRACT

Glucose-induced insulin secretion from pancreatic β-cells depends on mitochondrial activation. In the organelle, glucose-derived pyruvate is metabolised along the oxidative and anaplerotic pathway to generate downstream signals leading to insulin granule exocytosis. Entry into the oxidative pathway is catalysed by pyruvate dehydrogenase (PDH) and controlled in part by phosphorylation of the PDH E1α subunit blocking enzyme activity. We find that glucose but not other nutrient secretagogues induce PDH E1α phosphorylation in INS-1E cells and rat islets. INS-1E cells and primary β-cells express pyruvate dehydrogenase kinase (PDK) 1, 2 and 3, which mediate the observed phosphorylation. In INS-1E cells, suppression of the two main isoforms, PDK1 and PDK3, almost completely prevented PDH E1α phosphorylation. Under basal glucose conditions, phosphorylation was barely detectable and therefore the enzyme almost fully active (90% of maximal). During glucose stimulation, PDH is only partially inhibited (to 78% of maximal). Preventing PDH phosphorylation in situ after suppression of PDK1, 2 and 3 neither enhanced pyruvate oxidation nor insulin secretion. In conclusion, although glucose stimulates E1α phosphorylation and therefore inhibits PDH activity, this control mechanism by itself does not alter metabolism-secretion coupling in INS-1E clonal β-cells.
E1α subunit of PDH on Ser293 (site 1), Ser300 (site 2) and Ser232 (site 3) leading to the inactivation of the PDH complex (for review see [14–16]). Four PDK isoforms have been identified in mammalian tissues, several of which are expressed in pancreatic islets [17,18].

Dephosphorylation of E1α and, as a consequence, reactivation of PDH is catalysed by pyruvate dehydrogenase phosphatases (PDP) 1 or 2. Both phosphatases require Mg2+ while only PDP1 is activated by Ca2+ [19]. In the pancreatic β-cell, mitochondrial Ca2+ rises transiently shortly after nutrient stimulation and is an essential signal for the amplification of insulin secretion [20]. Mitochondrial Ca2+ could exert its signaling role at least in part through the activation of PDP1-mediated dephosphorylation of PDH.

To date, there is no consensus as to the activity of PDH under resting or nutrient-stimulated conditions in the pancreatic β-cell. While some studies find very low activity (<10%) under resting conditions and strong glucose-induced activation, other results describe constitutively high PDH activity (up to 94% of total) [18,21–23]. In addition to the still debated acute effect of glucose, chronic incubation of islets in high glucose or palmitate inhibits PDH due to increased PDK activity in the islets [18,24]. It is of interest, in this context, that glucose which promotes activation of hypoxia-inducible factor 1α (HIF1α) in islets by lowering intracellular oxygen tension also up regulates PDK1 expression [20].

Over-expression of either PDKs or PDPs has been used as an approach to assess the relevance of PDH phosphorylation as a regulatory mechanism during nutrient-stimulated insulin secretion. Over-expression of PDK3 or PDK4 clearly reduced PDH activity without lowering GSIS in rat islets or INS-1 cells [12,23]. Reducing PDH activity therefore does not appear sufficient to impair metabolism-secretion coupling. On the other hand, expressing a catalytically active form of PDP elevated PDH activity but failed to alter GSIS in rat islets [23]. In contrast to these negative findings, a recent study has observed enhanced GSIS after selective knockdown of PDK1 in INS-1 832/13 clonal β-cells [26]. PDK1 knockdown raised PDH activity, mitochondrial respiration and by favoring anaplerosis several intermediates of the TCA cycle.

Here we examined the expression of the different PDK isoforms as well as PDH phosphorylation in INS-1E and primary β-cells. We also studied whether PDH E1α phosphorylation exerts a control function in GSIS following knockdown of the different PDH isoforms expressed in INS-1E cells.

2. Materials and methods

2.1. Reagents

Chemicals were from either Sigma or Fluka (Buchs, Switzerland) unless otherwise indicated.

2.2. Preparation and culture of rat islets and FACS-purified α- and β-cells

Animal care and experimentation were conducted according to the guidelines of the Swiss Academy of Medical Sciences and performed with the permission of the Canton of Geneva Veterinary Office. Rat islet isolation and purification of islet cells were carried out as described previously [27].

2.3. Cell culture conditions

INS-1E cells [28] were cultured at 37 °C in humidified atmosphere (5% CO2) in RPMI-1640 medium containing 11 mM glucose (Invitrogen), supplemented with 10 mM Hepes, 5% (v/v) heat-inactivated fetal calf serum (Bruschwig AG, Switzerland), 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 50 μg/ml penicillin and 100 μg/ml streptomycin (INS medium). Most experiments were performed in Krebs-Ringer bicarbonate Hepes buffer (KRBH): 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 1.5 mM CaCl2, 10 mM Hepes, 5 mM NaHCO3, pH 7.4.

2.4. Analysis of gene expression

Total RNA was extracted using RNeasy Mini Kit (Qiagen). Samples were treated with 0.5 U of RNase-free DNase I (Ambion, Austin, TX, USA)/1 μg of RNA. Single-stranded DNA was synthesised employing SuperScript™ II reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen). Single-stranded DNA was used as a template in quantitative PCR using FastStart Universal SYBR Green mix (Roche Diagnostics GmbH, Mannheim, Germany) and specific primer pairs (Table 1). The results were analysed using ABI PRISM 7000 SDS software (Applied Biosystems, Foster City, CA, USA). Expression levels were calculated according to the Pfaffl method [29].

2.5. siRNA transfection

INS-1E cells were plated in antibiotic-free INS medium and transfected the next day with 30 nM siRNA and 1.4 μl/ml DharmaFECT 1 transfection reagent (Thermo Fisher Scientific, Lafayette, CO, USA), 24 h after transfection the medium was changed to regular INS medium. Quantitative RT-PCR was performed 96 h post-transfection. siRNAs were purchased from Ambion (Austin, TX, USA). siRNA sense strand sequences (5’→3’) are: PDK1: GAA CUG UUC AAG AAC GCA ATT; PDK2: AGA ACA UCC AGU ACU UUU UTT; PDK3: GAU CCA CGC GUU UUA GAU ATT; negative control siRNA #2 (Ambion).

2.6. Cell lysate preparation and Western blotting

INS-1E cells were washed once with ice-cold PBS without Ca2+ and Mg2+, harvested, extracted using lysis buffer (20 mM Tris–HCl, 2 mM EDTA, 2 mM EGTA, 0.5 mM PMSF, 10 mM NaF, 0.04% (v/v) β-mercaptoethanol, 1% (v/v) Triton X-100) and sonicated. Protein samples were separated on 10% SDS polyacrylamide gels and transferred to Hybond-ECL membranes (Amersham Biosciences).

Membranes were blocked in 4% fat-free powdered milk in TBS-T (25 mM Tris, 150 mM NaCl, 2.68 mM KCl, 0.1% (v/v) Tween-20, pH 7.4) for 50 min at room temperature and incubated at 4 °C overnight with a sheep polyclonal antibody against PDH E1α subunit phosphorylated on site 1 (Ser293) or site 2 (Ser300) (1 μg/ml in 3% milk in TBS-T). Secondary antibody: HRP-labeled anti-sheep antibody (DakoCytomation, Glostrup, Denmark; 0.43 μg/ml in 3% milk in TBS-T). Bands were detected using ECL chemiluminescent system (Amersham Biosciences). The membranes were re-probed using an antibody recognizing PDH E1α. The PDH antibodies [30] were kindly provided by Dr. H. Pilegaard (University of Copenhagen, Denmark).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used for quantitative RT-PCR. Sequences shown are 5’→3’.</th>
</tr>
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<tbody>
<tr>
<td>Gene name</td>
<td>Forward primer</td>
</tr>
<tr>
<td>Cyclophilin</td>
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</tr>
<tr>
<td>RPS-29</td>
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<td>PDK1</td>
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<td>GCT GTC CAT GAA GCA CTA TCT AGA</td>
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<tr>
<td>PDK3</td>
<td>TGA CCT AGG TGG TGG AGT AGC A</td>
</tr>
<tr>
<td>PDK4</td>
<td>TGT AAT GCG GCC AGA ATT G</td>
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2.7. PDH activity assay

PDH activity was measured using a PDH enzyme assay kit (MitoSciences, Eugene, OR, USA) according to the manufacturer’s protocol with the following modifications. During cell lysis and PDH immunocapture: 1 mM PMSF, protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), 1 mM EDTA, 20 mM NaF, 5 mM DCA was included. 1 mg of total cell lysate was loaded per anti-PDH antibody coated microtiter well. Enzyme activity was followed for 1 h at 25 °C on a FLUostar OPTIMA microplate reader (BMG Labtechnologies, Offenburg, Germany). To determine the maximal activity of fully-dephosphorylated PDH, parallel samples were treated with pyruvate dehydrogenase phosphatase PDP1 (MitoSciences, Eugene, OR, USA).

2.8. [1-14C]pyruvate oxidation measurements

INS-1E cells were plated in 6-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 0.5 × 10⁶ cells/well and grown for 48 h. Cells were switched for 2 h to RPMI-1640 medium containing 4 mM glucose and 1% (v/v) FCS. The cells were washed once and grown for 48 h. Cells were switched for 2 h to RPMI-1640 medium containing 4 mM glucose and 1% (v/v) FCS. The day after plating, cells were transfected with Hoechst 33342 dye (5 µg/ml) for 5 min at room temperature. Propidium iodide positive per total Hoechst stained nuclei. For each condition more than 6000 cells were analysed.

2.9. Cell death assays

Relative changes in cell death in INS-1E cell populations were measured using a Cell Death Detection ELISAPLUS kit (Roche Diagnostics GmbH, Mannheim, Germany). The percentage of apoptotic plus necrotic cells was determined using propidium iodide. Cells were incubated in KRBH buffer containing 5 mM glucose plus 0.1 mM cold pyruvate and 0.02 nCi/ml of [1-14C]pyruvate (American Radiolabeled Chemicals). During the incubation 14CO2 was captured with NaOH-soaked Whatman filters. To extract 14C from the medium 5 M HCl was injected. The captured 14C on filters was counted in scintillation vials (Wheaton, Millville, NJ, USA). The scintillation fluid was from Packard (USA).

2.10. Insulin secretion assay

INS-1E cells were plated into polyornithin-treated 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 7.5 × 10⁴ cells/well. The day after plating, cells were transfected with siRNAs. Secretion assays were performed 96 h later. Static insulin secretion assays and insulin measurements were carried out as described previously [31].

2.11. Mitochondrial Ca²⁺ measurements

INS-1E cells (7.5 × 10⁴ cells) were plated on polyornithin-treated 15 mm diameter Thermanox coverslips (Nalge Nunc, Rochester, NY, USA) in 4-well tissue culture plates. The day after plating, cells were transfected with siRNAs. 48 h post-transfection cells were infected for 2 h with adenovirus expressing mitochondrially-targeted aequorin under the control of the rat insulin promoter (Ad-RIP-mitoAequorin). 96 h post-transfection cells were transferred for 2 h to RPMI-1640 medium containing 4 mM glucose, 1% (v/v) FCS and 5 µM coelenterazine (Calbiochem). INS-1E cell perifusions and analysis of the mitoAequorin signal were carried out as described previously [20].

3. Results

3.1. Glucose stimulates PDH E1α phosphorylation in INS-1E cells and rat pancreatic islets

Enhanced mitochondrial oxidative metabolism following glucose stimulation of the pancreatic β-cells could in part be due to Ca²⁺-dependent dephosphorylation causing activation of PDH [22,32]. To test this possibility, we followed PDH phosphorylation in β-cells during acute glucose stimulation by Western blotting using an antibody specifically recognizing Ser²⁹³ (site 1) phosphorylated PDH E1α [30]. Only a faint band was detected in lysates prepared from INS-1E cells maintained at resting glucose concentrations (2.5 mM) suggesting that only a small fraction of total PDH is phosphorylated. Within 2 min following stimulation with 16.7 mM glucose, PDH E1α phosphorylation was markedly increased. Thereafter phosphorylation augmented further and remained elevated in the continued presence of glucose (Fig. 1A; up to 30 min). Over the time-course studied total PDH E1α protein levels on the other hand remained constant.

The minimal glucose concentration required for PDH E1α phosphorylation was 7.5 mM (Fig. 1B). Glucose-dependent phosphorylation was also observed on phosphorylation site 2 of PDH E1α as detected using an antibody specifically recognizing phosphorylated E1α (Ser³⁰⁰) (Fig. 1C). The concentration required to induce phosphorylation of PDH suggests that this posttranslational modification...
is linked to glucose metabolism and occurs at glucose levels similar to those necessary to induce GSIS.

Acute glucose-dependent phosphorylation of E1α was also observed in cultured rat islets (Fig. 1D). Glucose-dependent phosphorylation of PDH therefore occurs both in INS-1E and primary β-cells.

3.2. Ca²⁺ stimulates PDH E1α dephosphorylation in INS-1E cells

The above findings are counterintuitive as the mitochondrial Ca²⁺ rise associated with glucose stimulation in the β-cell would be expected to cause dephosphorylation and activation of PDH [20,33]. To assess whether Ca²⁺ promotes PDH dephosphorylation as observed in other cell types, we manipulated the glucose-associated Ca²⁺ rise. Removal of extracellular Ca²⁺ using EGTA abolishes both cytosolic and mitochondrial Ca²⁺ transients [20,34]. Suppression of Ca²⁺ signals with EGTA resulted in glucose-dependent PDH E1α phosphorylation, which was much more pronounced than with 16.7 mM glucose alone (Fig. 2A). Conversely, when voltage-dependent Ca²⁺ influx was potentiated by depolarizing the plasma membrane using 30 mM KCl, glucose-induced phosphorylation was fully suppressed (Fig. 2A). Under resting conditions, raising or lowering extracellular glucose concentrations (7 mM) glucose concentrations (Fig. 2B).

To assess dephosphorylation and activation of PDH E1α phosphorylation, we first measured the expression of different secretagogues as shown in the figure, followed by the addition of rotenone (rtn, 5 mM) for the indicated time. (A) Stimulation of INS-1E cells with 16.7 mM glucose and either EGTA (1 mM) or KCl (30 mM). (B) Cells were preincubated for 15 min with KRBH containing 2.5 mM glucose+5 mM leucine. (C) INS-1E cells were incubated for 15 min in KRBH containing 2.5 mM glucose+5 mM mm-succinate. (D) Cells were preincubated for 30 min in KRBH containing 2.5 mM glucose and then stimulated as indicated in the figure, followed by the addition of rotenone (rtn, 5 mM) for the indicated time. (C, D, E) INS-1E cells were incubated in KRBH containing different secretagogues as shown in the figure. (F) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose+5 mM dimethyl-glutamate or KRBH containing 2.5 mM glucose+5 mM DCA. The cells were then incubated for 15 min in KRBH containing 2.5 or 16.7 mM glucose for either low (2.5 mM) or intermediate (7 mM) glucose concentrations (Fig. 2B).

3.3. Alternative nutrient secretagogues do not induce PDH phosphorylation

Glucose may share some but not all downstream metabolites with other nutrient secretagogues. Leucine and monomethyl succinate for instance bypass glycolysis and do not depend on PDH activity for their metabolism. Leucine provides mitochondrial acetyl-CoA as a substrate for the TCA cycle while monomethyl succinate principally metabolizes pyruvate dehydrogenase kinases and Ca²⁺-activated PDH phosphatase. INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose and then stimulated as indicated in the figure. The cell lysates (50 μg) were analysed with the antibody recognizing site 1 phosphorylated PDH E1α (p-Ser293) (upper panels) and re-probed with an antibody raising against total PDH E1α (lower panels). (A) Stimulation of INS-1E cells with 16.7 mM glucose and either EGTA (1 mM) or KCl (30 mM). (B) Cells were preincubated for 15 min with KRBH containing glucose as shown in the figure, followed by the addition of rotenone (rtn, 5 mM) for the indicated time. (C, D, E) INS-1E cells were incubated in KRBH containing different secretagogues as shown in the figure. (F) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose or KRBH containing 2.5 mM glucose+5 mM DCA. The cells were then incubated for 15 min in KRBH containing 2.5 or 16.7 mM glucose with or without 5 mM DCA as indicated.

3.4. PDK isoforms are required for glucose-induced PDH phosphorylation in β-cells

Dichloroacetate (DCA), a general inhibitor of PDKs, prevented glucose-induced PDH E1α Ser293 phosphorylation (Fig. 2F). In order to identify the PDK isoform responsible for glucose-induced PDH phosphorylation, we first measured the expression of different secretagogues: PDK1, PDK2, PDK3 and PDK4. mRNA levels in rat islets, purified β-cells and INS-1E cells were compared to purified α-cells and a panel of rat tissues by quantitative RT-PCR (Fig. 3A–D). PDK1 expression in β- or INS-1E cells was comparable to most tissues tested (Fig. 3A) but about 10-fold lower than in the heart, which is known to express high levels of this isoform [38]. Weak expression of PDK2 mRNA was observed in both β- and INS-1E cells (more than 10 times lower than in highly expressing tissues such as kidney, brain and heart; Fig. 3B). PDK3, on the other hand, was well expressed in islet α- and β-cells as well as INS-1E cells. PDK3 expression levels were more than 10 times higher than in poorly expressing tissues such as liver and heart (Fig. 3C). In contrast, PDK4 expression was almost undetectable in INS-1E cells but expressed in α-cells and intact islets (Fig. 3D). In purified primary β-cells, expression was
lower than in intact islets suggesting that PDK4 expression originates from contaminating non-β-cells.

PDK expression can be regulated by factors such as growth and nutrient conditions [17,18]. For direct comparison, expression of PDKs in INS-1E cells was thus also studied under conditions matching those shown in Fig. 1A. RNA was isolated from INS-1E cells preincubated in KRBH 2.5 mM glucose and increasing times at either 2.5 or 16.7 mM glucose. PDK1, 2 and 3 expression was unaffected in response to acute glucose stimulation (Fig. 3E). The observed glucose-induced phosphorylation of PDH is therefore not due to the induction of PDK expression. PDK4 was not or only barely detectable in all INS-1E cell samples studied. Based on this analysis, PDK4 is unlikely to be of importance for PDH regulation in INS-1E cells.

3.5. PDK1 and PDK3 phosphorylate PDH E1α in INS-1E cells

To test the contribution of the PDKs to glucose-induced PDH phosphorylation in insulin secreting cells, we specifically knocked down the expression of PDK isoforms. siRNAs designed to target PDK1, PDK2 or PDK3 reduced the respective mRNA levels by 58.8%, 64.6% and 73.6% compared to control (Fig. 4A). The reduction of individual PDKs neither resulted in the concomitant decrease nor a compensatory increase in expression of the other PDK isoforms (data not shown).

Knockdown of PDK1 or PDK3 led to a marked reduction of glucose-dependent PDH E1α phosphorylation in INS-1E cells (Fig. 4B). In comparison, lowering PDK2 had little or no effect on...
mitochondrial Ca\textsuperscript{2+} response in control siRNA-treated INS-1E cells. Preincubated for 30 min in KRBH containing 2.5 mM glucose followed by 15 min incubation in KRBH containing either 2.5 or 16.7 mM glucose. Cell lysates (50 μg) were analysed by Western blotting using the antibody recognizing site 1 phosphorylated PDH E1 (α-p-Ser\textsuperscript{293}) (upper panel) and re-probed with an antibody raised against total PDH E1 (lower panel).

Fig. 4. siRNA-dependent lowering of PDK expression. (A) INS-1E cells were transfected without (mock; transfection reagent only), control siRNA (siRNA #2; Ambion) or siRNAs specifically targeting PDK1, PDK2 or PDK3 as shown in the figure. 96 h after transfection, expression of PDK1, PDK2 and PDK3 was measured by quantitative RT-PCR. Expression levels are displayed relative to non-treated INS-1E cells. Values represent the mean of three independent experiments ±/− SEM. Statistical significance was determined by Student’s t-test for unpaired data; *p<0.05, **p<0.01. (B) INS-1E cells were transfected with control siRNA, siRNA against PDK1, PDK2 or PDK3 or combinations of siRNAs as indicated in the figure. 96 h after transfection cells were preincubated for 30 min in KRBH containing 2.5 mM glucose followed by 15 min incubation in KRBH containing either 2.5 or 16.7 mM glucose. Cell lysates (50 μg) were analysed by Western blotting using the antibody recognizing site 1 phosphorylated PDH E1α (α-p-Ser\textsuperscript{293}) (upper panel) and re-probed with an antibody raised against total PDH E1α (lower panel).

3.6. PDH E1α phosphorylation does not affect glucose-stimulated insulin secretion

Phosphorylation of PDH may be inhibitory for metabolism-secretion coupling as it attenuates pyruvate oxidation. Alternatively, PDH phosphorylation could stimulate metabolism-secretion coupling by favoring anaplerosis and the generation of metabolic signals. Therefore we followed metabolism-secretion coupling after knockdown of individual PDKs or a combination of PDK isoforms (Fig. 5A). Control INS-1E cells exposed for 30 min to 16.7 mM glucose increased hormone secretion 3.1-fold compared to 2.5 mM glucose. Lowering the expression of the PDK1, PDK2 or PDK3 did neither affect basal insulin secretion nor the glucose-induced response. Glucose-stimulated insulin secretion was even preserved under conditions that almost completely prevented PDH phosphorylation, using siRNAs against PDK1 and PDK3 or siRNAs against all three studied PDK isoforms (Fig. 5A). During GSIS cytosolic Ca\textsuperscript{2+} rises are relayed into mitochondria. The Ca\textsuperscript{2+} rise in the organelle can be used as a sensitive kinetic read-out of the underlying metabolism-secretion coupling [20]. Glucose initiated a robust mitochondrial Ca\textsuperscript{2+} response in control siRNA-treated INS-1E cells. Down-regulation in combination of PDK1, PDK2 and PDK3 did neither delay the time of onset nor the amplitude of the glucose-induced mitochondrial Ca\textsuperscript{2+} rise (Fig. 5B). Taken together, metabolism-secretion coupling is normal under conditions that prevent glucose-induced phosphorylation of PDH E1α in INS-1E cells.

3.7. Glucose-stimulated PDH E1α phosphorylation decreases PDH activity

Based on the preceding results, we assessed the effect of phosphorylation on PDH activity. Enzyme activity was determined in extracts from INS-1E cells maintained for 15 min in either resting (2.5 mM) or stimulatory glucose (16.7 mM). Half of the sample was used to determine total PDH activity by treating the lysate with PDP1. Results are expressed as percentage of total activity. At 2.5 mM glucose 91 % of PDH was active consistent with the faint signal obtained using phosphospecific PDH antibodies. Following stimulation with 16.7 mM glucose PDH activity was reduced to 78% of total (Fig. 6A). The remaining large fraction of active PDH may leave pyruvate metabolism close to normal thus not affecting metabolism-secretion coupling under our standard conditions. This was tested in intact cells measuring [\textsuperscript{1-14C}] pyruvate oxidation. Release of \textsuperscript{14CO2} as PDH decarboxylates \textsuperscript{1-14C} pyruvate is an in situ read-out for PDH activity. INS-1E cells were incubated in the presence of a constant amount of labeled and cold pyruvate (0.1 mM). When INS-1E cells were incubated in 16.7 mM glucose, oxidation of labeled pyruvate was decreased by ~30% compared to the 2.5 mM glucose control (Fig. 6B). Mechanically, there are at least two possible ways to explain reduced \textsuperscript{1-14C} pyruvate oxidation: 1) PDH phosphorylation reduces pyruvate oxidation as demonstrated in Fig. 6A. 2) Glucose metabolism forms cold pyruvate to the extent that it competes with the constant amount of labeled pyruvate added. To assess the contribution of PDH phosphorylation on pyruvate flux we therefore compared pyruvate oxidation under identical conditions but after suppression of PDH phosphorylation using PDK siRNAs. For all conditions tested glucose lowered pyruvate oxidation (Fig. 6C). Interestingly, at 16.7 mM glucose pyruvate oxidation was unaffected whether or not PDH phosphorylation was suppressed by siRNAs against PDK1, PDK2 and PDK3 (Fig. 6C). Our
results show that PDH activity is lowered to 78% of maximal by glucose-induced phosphorylation but is still sufficient to maintain normal pyruvate flux. Under the conditions studied here, the observed PDH E1α phosphorylation therefore does not impact on metabolism-secretion coupling.

3.8. PDH E1α phosphorylation is not important for INS-1E cell viability during glucotoxicity

Rather than playing an important role in acute glucose-stimulated insulin secretion, PDH phosphorylation may be able to adapt β-cell function to the average glucose availability. For instance, PDH phosphorylation may have some protective function when glucose concentrations remain continuously high. In support of this possibility several PDK isoforms are upregulated in islet β-cells cultured under glucotoxic conditions [18]. In agreement with these earlier findings, we observed that PDH E1α phosphorylation in INS-1E cells was dramatically increased when the cells were cultured for 48 h in 30 mM glucose (Fig. 7A) compared to culture in standard medium (11 mM glucose). After lowering the glucose concentration in the medium to 4 mM for 2 h, PDH E1α phosphorylation was undetectable showing that this glucose effect is reversible. Interestingly, INS-1E cells cultured for 2 days in 30 mM glucose medium demonstrated more robust PDH E1α phosphorylation during acute glucose stimulation, compared with cells cultured in 11 mM glucose (Fig. 7B). To elucidate whether more robust glucose-induced PDH phosphorylation is due to an elevated expression of PDKs in INS-1E cells, we cultured the cells for 48 h in 11 respectively 30 mM glucose medium (condition as described for Fig. 7A). However, the glucotoxic (30 mM glucose) condition did not alter PDK 1–3 expression and PDK4 remained barely detectable (Fig. 7C). Enhanced PDH phosphorylation in INS-1E cells is not due to changes in PDK expression but likely due to other adaptive changes.

Prolonged exposure of β-cells to elevated glucose involves activation of pro-apoptotic and lipogenic genes, resulting in increased lipid accumulation, reduced GSIS and apoptosis [39,40]. Enhanced PDH E1α phosphorylation, as observed here, should favor anaplerotic flux resulting in export of mitochondrial substrates for fatty acid synthesis. This may reduce β-cell function and render them more susceptible to cell death. We therefore tested whether control of PDH activity by phosphorylation influenced apoptosis of INS-1E cells exposed to gluotoxic conditions (48 h; 30 mM glucose). Consistent with earlier results, cell death as assessed by the release of histone-complexed DNA fragments (Fig. 7D) or the fraction of dead cells (Fig. 7E; propidium iodide permeable cells; see Materials and methods). Half of each sample was treated with PDP1 in order to determine total PDH activity. Results are expressed as percentage of total PDH activity. Values are the mean of four independent experiments +/−SEM. (A) INS-1E cells were transfected with control siRNA, siRNA against PDK1, PDK2 or PDK3 or combinations of siRNAs as indicated in the legend. (B) INS-1E cells were transfected with control siRNA, siRNA against PDK1, PDK2 or PDK3 or combinations of siRNAs as indicated. 96 h post-transfection mitochondrial calcium, nM was measured in INS-1E cells transfected with siRNA (grey trace) or a combination of siRNAs targeting PDK1, PDK2 and PDK3 (grey trace) and infected with adenovirus expressing mitochondrially-targeted aequorin. Cells were perifused with KRBH containing the indicated glucose concentrations. At the end of the experiment Ca2+ influx was triggered using 30 mM KCl. Values represent the mean of five independent experiments. SEM are shown every 60 s. (C) INS-1E cells were transfected with siRNAs as indicated. 96 h post-transfection [1-14C] pyruvate oxidation was measured as in (B); 2.5 mM glucose (grey bars), 16.7 mM glucose (black bars). Values represent the mean of three independent experiments +/−SEM. Statistical significance was determined by Student’s t-test for unpaired data: *p=0.05, **p=0.01.

Fig. 6. Glucose-dependent PDH phosphorylation decreases PDH activity but not pyruvate flux in INS-1E cells. (A) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose followed by 15 min in KRBH containing 2.5 or 16.7 mM glucose. Cells were collected, lysed and PDH activity was measured as described in Materials and methods. Half of each sample was treated with PDP1 in order to determine total PDH activity. Results are expressed as percentage of total PDH activity. Values are the mean of four independent experiments +/−SEM. (B) INS-1E cells were preincubated for 30 min in KRBH 2.5 mM glucose. The cells were then washed in KRBH without glucose and incubated with KRBH 2.5 or 16.7 mM glucose containing 0.1 mM cold pyruvate and [1-14C] pyruvate as described in Materials and methods. Data are expressed as percentage of pyruvate oxidation at 2.5 mM glucose. (C) INS-1E cells were transfected with siRNAs as indicated. 96 h post-transfection [1-14C] pyruvate oxidation was measured as in (B); 2.5 mM glucose (grey bars), 16.7 mM glucose (black bars). Values represent the mean of three independent experiments +/−SEM. Statistical significance was determined by Student’s t-test for unpaired data: *p=0.05, **p=0.01.

Fig. 5. Suppression of PDH E1α phosphorylation does not alter metabolism-secretion coupling. INS-1E cells were transfected with control siRNA, siRNA against PDK1, PDK2 or PDK3 or combinations of siRNAs as indicated in the figure. Experiments were performed 96 h after siRNA transfection. (A) Cells were preincubated for 30 min in KRBH containing 2.5 mM glucose followed by 30 min in KRBH containing 2.5 mM (grey bars) or 16.7 mM glucose (black bars). Insulin secretion and content were measured. Secreted insulin is expressed as percentage of insulin content. Values represent the mean of four independent experiments. SEM are shown every 60 s.

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Fig. 7. PDH E1α phosphorylation is enhanced after culture in glucotoxic conditions but does not affect cell viability. (A) INS-1E cells were incubated for 48 h in INS medium containing 11 or 30 mM glucose. Cell lysates were prepared immediately after medium removal (lanes 1 and 3) or after lowering glucose to 4 mM for 2 h (lanes 2 and 4). Cell lysates (50 μg) were analysed by Western blotting using the antibody recognizing site 1 phosphorylated PDH E1α (p-Ser292) (upper panel) and re-probed with an antibody raised against total PDH E1α (lower panel). (B) INS-1E cells were incubated for 48 h in INS medium containing 11 or 30 mM glucose. The cells were then switched for 30 min to KRBH containing 2.5 mM glucose followed by 15 min incubation in KRBH with the indicated glucose concentrations. Cell lysates (50 μg) were analysed as described in A. (C) Expression of PDK isoforms was measured by quantitative RT-PCR in INS-1E cells grown in regular medium (grey bars) and compared to cells grown in medium containing 30 mM glucose (black bars) for 48 h (condition as in panel A lanes 1 and 3). Shown are average CT values from 3 independent experiments performed in triplicate (+/−SEM). The glucose concentration in the medium did not significantly affect PDK1, 2 and 3 expression, PDK4 was not detectable for either glucose concentration. (D, E) INS-1E cells were transfected with siRNAs as indicated. 48 h post-transfection the cells were changed to medium containing either 11 mM (grey bars) or 30 mM glucose (black bars). Cell death was measured as an increase in histone-complexed DNA fragments (D) or the fraction of dead cells (E; propidium iodide permeable cells) 48 h later as described in Materials and methods. Values represent the mean of three independent experiments +/−SEM.

4. Discussion

In the β-cell, PDH serves a regulatory function as its activity determines to what extent glucose-derived pyruvate enters the oxidative or anaplerotic pathways. As both branches of metabolism are necessary for GSIS, the impact of PDH regulation on metabolism-secretion coupling is difficult to predict. Unfortunately, there is a large discrepancy between different publications concerning the fraction of active PDH in β-cells. At sub-stimulatory glucose concentrations PDH activity in islets was found to be 5%, 16%, 40% or 94% of total [18,21–23]. Depending on the study, glucose thus caused no or very pronounced changes in PDH activity. Nevertheless, glucose-dependent phosphorylation of PDH was unexplored. The well described glucose-induced mitochondrial Ca2+ rise should activate PDP1 and therefore favor PDH dephosphorylation. We show in intact cells that elevation of mitochondrial Ca2+ indeed favors dephosphorylation of PDH E1α.

For each nutrient condition a balance may exist between PDH E1α phosphorylation and dephosphorylation. Reciprocal regulation of PDK and PDP activities has already been reported for instance in rat heart and kidney [42]. As glucose raises matrix Ca2+ and therefore stimulates PDP activity, net phosphorylation of PDH E1α can only be explained if activation of PDK is more pronounced than stimulation of PDP. Such stimulation of PDK is likely due to signals linked to glucose metabolism. For example, the NADH/NAD+, acetyl-CoA/CoA and ATP/ADP ratios, which rapidly increase following glucose stimulation of β-cells [43,44], are known to activate PDK [45]. However, artificially raising the mitochondrial NADH/NAD+ ratio using the complex I inhibitor rotenone did not promote PDH E1α phosphorylation. The changes required to induce PDH E1α phosphorylation by glucose therefore must be the consequence of the combination of several PDK activators.

Glucose also gives rise to the PDK inhibitor pyruvate, which increases several fold during glucose stimulation of INS-1 cells [46]. INS-1E cells, unlike primary β-cells, express the monocarboxylate
transporter and pyruvate (5 mM) directly stimulates insulin secretion [47]. Nevertheless, pyruvate failed to cause phosphorylation of PDH E1α (data not shown). Under these conditions, intramitochondrial pyruvate may reach sufficiently high concentrations to inhibit PDKs.

Phosphorylation of PDH E1α during glucose stimulation is consistent with the concept that PDH activity is inhibited when energy is abundant (high ATP/ADP ratio) and consequently the rate of glucose oxidation should be reduced. It was therefore very surprising that other secretagogues such as leucine and monomethyl succinate, did not cause PDH E1α phosphorylation. These nutrients bypass glycolysis and PDH but their effects on mitochondrial oxidative metabolism, respiration and mitochondrial signals resemble many of the changes elicited by glucose. A search for candidate metabolites required for glucose-induced PDH E1α phosphorylation will have to focus on metabolites specifically linked to glucose metabolism. One such metabolite is glutamate [13,36,46], which we tested using the membrane-permeable dimethyl glutamate. However, intracellular glutamate caused only weak phosphorylation of PDH E1α over a time-course much slower than glucose-induced phosphorylation. Thus, the glucose effect cannot solely be mediated by glutamate. It may be speculated that in order to cause PDH E1α phosphorylation a nutrient must fulfill at least two requirements. First, it should raise the metabolite ratios that activate PDKs and second, provide the substrate pyruvate. We speculate that in situ activated PDKs only recognise and therefore phosphorylate PDH which is actively turning over its substrate. These two criteria are only met by glucose but not nutrient secretagogues that bypass glycolysis.

PDH E1α phosphorylation was prevented by the pyruvate analogue DCA demonstrating that glucose acts through the activation of PDKs. We examined the expression of PDKs in INS-1E cells, purified β- and α-cells as well as intact rat islets. In agreement with earlier studies we detected expression of PDK1, PDK2 and PDK4 in intact islets [48]. Through the study of purified islet cells we find PDK4 to be preferentially expressed in α-cells. PDK4 was close to undetectable in INS-1E cells. The low level of PDK4 expression in β-cells may therefore be due to contaminating non-β-cells. These results are supported by expression profiling in β-cells obtained after laser capture, in which PDK4 expression was not detected [49]. Surprisingly, we find robust expression of PDK3 in rat β-cells, while this isoform was not detected in mouse islets [18]. In support of our expression data, PDK3 like PDK1 strongly contributes to glucose-induced phosphorylation of PDH E1α, while the contribution of PDK2 was only modest.

Suppression of PDK1 has recently been found to enhance GSIS [26]. Our prediction therefore was that PDH phosphorylation limits the acute glucose response. Alternatively, we could have expected that phosphorylation plays a role in accelerating anaplerosis and if prevented would lower amplification of insulin secretion. Preventing glucose-induced PDH phosphorylation did not affect insulin secretion even when siRNAs against PDK 1, 2 and 3 were combined. Our results therefore are in agreement with a study over-expressing the catalytic subunit of PDP in β-cell mitochondria, which did not result in changes in metabolism-secretion coupling [23].

Under the conditions studied here, PDH activity was reduced from 91% to 78% of total during the shift from low to stimulatory glucose, which did not impact on GSIS. Our experiments also show that oxidation of [1-13C] pyruvate is reduced by 30% when cells are shifted to high glucose, which agrees with glucose-induced PDH phosphorylation. Using siRNAs against PDK1, PDK2 and PDK3 we find that glucose still lowered oxidation of [1-13C] pyruvate in intact cells. We conclude that reduced oxidation of the labeled pyruvate occurs as it competes with cold pyruvate derived from glucose. Flux of pyruvate through the oxidative pathway is not lowered when the cells are stimulated by glucose despite the small reduction of PDH activity.

It is possible that PDH expression in INS-1E cells is in excess and therefore lowering its activity is of little consequence. Alternatively, reduced PDH activity may elevate the concentration of mitochondrial pyruvate to the point where pyruvate oxidation matches the rate observed in control cells. Liu and colleagues have proposed a similar mechanism to explain normal oxidative metabolism in β-cells with impaired PDH function [24].

It is noteworthy that islets from the β-cell-specific PDH (Pdh1a) knockout mouse still respond to glucose albeit with a lower fold change than controls [8]. Alternative pathways may take over in the mutant mice. The here observed PDH phosphorylation by glucose may protect from excessive glucose oxidation while having little impact on the acute regulation of GSIS.

In summary, our measurements demonstrate that in INS-1E cells PDH is almost maximally active and partially inhibited when stimulated with glucose. This effect is specific for the hexose as it was not observed with other nutrient secretagogues. The here described glucose-induced phosphorylation (inhibition) of PDH is not sufficient to slow pyruvate flux nor does it alter metabolism-secretion coupling in INS-1E clonal β-cells.

Acknowledgements

We thank N. Aebischer, D. Brighouse, E. Danenberg, E. Husi, D. Nappey and C. Bartley for expert technical assistance. We are grateful to Dr. H. Pilegaard (University of Copenhagen) for providing us with phospho-specific PDH antibodies. We are indebted to Prof. P. A. Halban (University of Geneva) and members of his group for providing FACS sorted islet cells. We further acknowledge the continued support by the Swiss National Foundation (310000-116750/1) and EuroDiab (LSHM-CT-2006-518153) a European–Community funded project under framework program 6.

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