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Reference

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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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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 barely 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.

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1. Introduction
Pancreatic β-cells adapt insulin secretion to changes in plasma nutrient concentrations. The exocytosis of insulin granules, the main function of this cell type, is induced by the uptake and metabolism of nutrient secretagogues. Mitochondria are a key organelle for nutrient metabolism and a source of signals that directly influence β-cell secretory function [1,2]. This is achieved in part through the oxidation of nutrients in the mitochondrial matrix, which provides reducing equivalents serving as substrate for oxidative phosphorylation. The associated activation of respiration promotes the synthesis and export of ATP from mitochondria. The resulting increase of the cytosolic ATP/ADP ratio favors closure of KATP channels. This leads to depolarization of the plasma membrane and thereby induces β-cell electrical activity (triggering pathway of insulin secretion). In addition, anaplerosis in β-cell mitochondria is able to increase the pool of TCA cycle intermediates several of which are exported to the cytosol where they may act as signals amplifying the insulin secretory response (amplifying pathway) [2,3].

Glucose-derived pyruvate is efficiently taken up by β-cell mitochondria presumably via the recently identified pyruvate carrier [4]. In the mitochondrial matrix pyruvate is oxidised by pyruvate dehydrogenase (PDH) to form acetyl-CoA. Alternatively pyruvate enters the anaplerotic pathway initiated by pyruvate carboxylase (PC). In the β-cell, both pathways are used about equally [5–7]. Interestingly, following disruption of the pyruvate dehydrogenase gene (Pdha1) in β-cells, glucose-stimulated insulin secretion (GSIS) was impaired but not abolished [8]. These results demonstrate that GSIS does not solely rely on mitochondrial pyruvate oxidation. These findings are consistent with the view that metabolites fluxes through both PDH and PC are important for insulin secretion [9,10]. Interfering with PC function reduces anaplerosis required for the cycling of metabolites across the inner mitochondrial membrane and lowers the cellular ATP/ADP ratio, therefore inhibiting GSIS [9–13].

In the β-cell, the relative use of the oxidative and anaplerotic pathways of mitochondrial pyruvate metabolism must be controlled and adjusted to the nutrient conditions. Indeed, anaplerosis of glucose-derived carbons increases as the glucose concentration rises [7,10]. Several important control mechanisms are decisive for the relative flux through PDH and PC. For instance elevation of the NADH/NAD⁺, acetylCoA/CoA or ATP/ADP ratios is inhibitory for PDH enzyme activity. Reversible phosphorylation is another mechanism controlling PDH activity. Pyruvate dehydrogenase kinases (PDK) phosphorylate PDH E1α, thereby almost completely inhibiting enzyme activity [14–16]. PDKs phosphorylate the...
E1α subunit of PDH on Ser\(^{293}\) (site 1), Ser\(^{300}\) (site 2) and Ser\(^{232}\) (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].

De-phosphorylation of E1α and, as a consequence, reactivation of PDH is catalysed by pyruvate dehydrogenase phosphatases (PDP) 1 or 2. Both phosphatases require Mg\(^{2+}\) while only PDP1 is activated by Ca\(^{2+}\) [19]. In the pancreatic β-cells, mitochondrial Ca\(^{2+}\) rises transiently shortly after nutrient stimulation and is an essential signal for the amplification of insulin secretion [20]. Mitochondrial Ca\(^{2+}\) 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 upregulates PDK1 expression [25].

Over-expression of either PDKs or PDHs 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 FACSPurified 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% CO\(_2\) in RPMI-1640 medium containing 11 mM glucose (Invitrogen), supplemented with 10 mM Hepes, 5% (v/v) heat-inactivated fetal calf serum (Brunschwig 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 (KRHB): 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH\(_2\)PO\(_4\), 0.5 mM MgSO\(_4\), 1.5 mM CaCl\(_2\), 10 mM Hepes, 5 mM NaHCO\(_3\), 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 Ca\(^{2+}\) and Mg\(^{2+}\)\(^{-}\), 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) 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 sheep polyclonal antibody against PDH E1α subunit phosphorylated on site 1 (Ser\(^{293}\)) or site 2 (Ser\(^{300}\)) (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 1

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciclophilin</td>
<td>CGT GGG CTC GGT TGT CTT</td>
<td>TGA CTT TAG GTC CTT TCT TCT TAT TG</td>
</tr>
<tr>
<td>RPS-29</td>
<td>CGT CCG TTA GTC CAA CTT AAT GAA G</td>
<td>GCT GAA CAT GTC CCG ACA GT</td>
</tr>
<tr>
<td>PDK1</td>
<td>CGG TGC CCC TGG TCG CAT TT</td>
<td>GGA TCC GTC CCG TAG CCC CC</td>
</tr>
<tr>
<td>PDK2</td>
<td>GCT GTC CAT GAA GCA GGA TCT TCT ACA</td>
<td>CGC AGG AGC GTC AAT GAA GTT TT</td>
</tr>
<tr>
<td>PDK3</td>
<td>TGA CCT AGC TGG TGG AGC ACT CCC A</td>
<td>ACC AAA TCC AGG CAA GGG AGC A</td>
</tr>
<tr>
<td>PDK4</td>
<td>TCT AAC GTC GCC AGA ATT ACA GC</td>
<td>GAA CTA AGC CTA GTC GGA TG</td>
</tr>
</tbody>
</table>

Primer sequences 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).
2.7. 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 Labotechnologies, 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 with KRBH 2.5 mM glucose and preincubated in the same buffer for 30 min. Labelling was performed for 1 h in KRBH containing 2.5 or 16.7 mM glucose plus 0.1 mM cold pyruvate and 0.02 nCi/ml of [1-14C] pyruvate (American Radiolabeled Chemicals). During the incubation period, the cells were incubated at 37 °C. To extract 14CO₂ from the medium 5 M HCl was injected. The captured 14CO₂ was captured with NaOH-soaked Whatman filters. To extract 14C on filters was counted in scintillation vials (Wheaton, Millville, NJ, USA). The scintillation fluid was from Packard (USA).

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 μg/ml propidium iodide for 20 min at 37 °C. The nuclei of all cells in the culture were then stained with Hoechst 33342 dye (5 μg/ml) for 5 min at room temperature. Fluorescence pictures were taken to calculate the percentage of propidium iodide positive per total Hoechst stained nuclei. For each condition more than 6000 cells were analysed.

2.10. Insulin secretion assay

INS-1E cells were plated onto polyornithin-coated 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 polyntrin-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 perfusions 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-cell 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 associated with a critical glucose concentration, which is 10-fold lower than the threshold concentration required for maximal mitochondrial oxidative metabolism.

![Image](file://url)

**Fig. 1.** Acute glucose-dependent PDH E1α phosphorylation in INS-1E cells and rat pancreatic islets. (A) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose and stimulated with 16.7 mM glucose for the indicated time. Cell lysates (50 μg) were analysed as described in (A).
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 suppressing Ca²⁺ signals did not alter PDH E1α phosphorylation (data not shown). The results demonstrate that in INS-1E cells, Ca²⁺ activates PDP similar to findings in other cell types. However, under physiological conditions, glucose shifts the equilibrium towards phosphorylation despite simultaneous Ca²⁺-activated dephosphorylation.

In addition to Ca²⁺, matrix metabolites are known to control PDH activity. For instance a high ratio of NADH/NAD+, such as observed after glucose stimulation, inhibits PDH activity. We therefore tested whether increasing NADH levels alone could mimic glucose-induced PDH phosphorylation. The complex I inhibitor rotenone was used to rapidly raise matrix NADH but this manipulation failed to influence PDH phosphorylation at 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 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 bypass glycolysis and do not depend on PDH activity for their metabolism.

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 isoforms: 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. 1 A. 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. 3 E). 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
glucose-induced phosphorylation of the enzyme. Using different combinations of siRNAs showed that PDH E1α phosphorylation is markedly decreased under all conditions where PDK1 or PDK3 are knocked down (Fig. 4B). The results also demonstrate that lowering of these PDKs cannot be compensated for by enhanced activity of the other isoforms. Combining siRNAs against all three expressed isoforms consistently resulted in the almost complete suppression of PDH phosphorylation. Taken together, all three PDK isoforms cannot be compensated for by enhanced activity of the other isoforms. Combining siRNAs against all three expressed isoforms resulted in the almost complete suppression of PDH phosphorylation. Taken together, all three PDK isoforms knocked down (Fig. 4B). The results also demonstrate that lowering of these PDKs cannot be compensated for by enhanced activity of the other isoforms. Combining siRNAs against all three expressed isoforms consistently resulted in the almost complete suppression of PDH phosphorylation. Taken together, all three PDK isoforms cannot be compensated for by enhanced activity of the other isoforms. Combining siRNAs against all three expressed isoforms resulted in the almost complete suppression of PDH phosphorylation. Taken together, all three PDK isoforms having the strongest impact.

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 Ca²⁺ rises are relayed into mitochondria. The Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 [1-14C] pyruvate oxidation. Release of 14CO₂ as PDH decarboxylates [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). Mechanistically, there are at least two possible ways to explain reduced [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 anaplerosis resulting in the 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 glucotoxic 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
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 death (relative to non-transfected) was measured as an increase in α-phosphorylated PDH E1α (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 activation of the enzyme.

Here we demonstrate that acute stimulation of rat islets or INS-1E insulinoma cells with glucose elicits rapid phosphorylation of PDH E1α. Under non-stimulatory conditions, however, PDH phosphorylation was barely detectable. In addition, PDH activity in INS-1E cells was almost maximal (91% active) at resting glucose concentrations and was reduced to 78% at a maximal stimulatory concentration. Moreover, our results were consistent using two independent methods. Our findings are in agreement with 13C-NMR experiments that observed elevated anaplerotic flux following glucose stimulation [10,41] as inhibition of PDH by phosphorylation should favor carboxylation of pyruvate by PC.

Nevertheless, glucose-dependent phosphorylation of PDH was unexpected. 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 4 (MCT4) which is the rat homolog of the human MCT1. Transporter expression is controlled by glucose concentration [47]. At glucose concentrations above the transport maximum, glucose is not able to enter the cell. Instead, the PDK inhibitor pyruvate is generated which might be responsible for the decrease in PDH activity.
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 PKDs.

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 PKDs and second, provide the substrate pyruvate. We speculate that in situ activated PKDs only recognize 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 PKDs. We examined the expression of PKDs in INS-1E cells, purified β- and α-cells as well as intact rat islets. In agreement with earlier studies we detected expression of PKD1, PKD2 and PKD4 in intact islets [48]. Through the study of purified islet cells we find PKD4 to be preferentially expressed in α-cells. PKD4 was close to undetectable in INS-1E cells. The low level of PKD4 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 PKD4 expression was not detected [49]. Surprisingly, we find robust expression of PKD3 in rat β-cells, while this isoform was not detected in mouse islets [18]. In support of our expression data, PKD3 like PKD1 strongly contributes to glucose-induced phosphorylation of PDH E1α, while the contribution of PKD2 was only modest.

Suppression of PKD1 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 anaerobiosis and if prevented would lower amplification of insulin secretion. Preventing glucose-induced PDH phosphorylation did not affect insulin secretion even when siRNAs against PKD 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 PKD1, PKD2 and PKD3 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 maximaly 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.

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