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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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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.

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 K_{ATP} 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 metabolite 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

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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].

Dephosphorylation 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-cell, 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 up regulates PDK1 expression [25].

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 PDK 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% 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 (KRBH): 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_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) 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 (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
Primers used for quantitative RT-PCR. Sequences shown are 5′→3′.

Gene name	Forward primer	Reverse primer
Cyclophilin	CGT GGG CTC CGT TGT CTT	TGA CTT TAG GTC CCT TCT TCT TAT CG
RPS-29	GGT CGC TTA GTC CAA CTT AAT GAA G	GCT GAA CAT GTG CCG ACA GT
PDK1	CGG TGC CCC TGG CTG GAT TT	GCA TCC GTC CCG TAG CCC TC
PDK2	GCT GTC CAT GAA GCA GTT TCT AGA	CGG AGG AAG GTG AAT GAA GTT TT
PDK3	TGA CCT AGG TGG TGG AGT CCC A	ACC AAA TCC AGC CAA GGG AGC A
PDK4	TCT AAC GTC GCC AGA ATT AAA GC	GAA CGT ACA CGA TGT GGA TTG G