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Mitochondrial phosphate transport during nutrient stimulation of INS-1E insulinoma cells

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ABSTRACT

Here, we have investigated the role of inorganic phosphate (P_i) transport in mitochondria of rat clonal β-cells. In α-toxin-permeabilized INS-1E cells, succinate and glycerol-3-phosphate increased mitochondrial ATP release which depends on exogenous ADP and P_i. In the presence of substrates, addition of P_i caused mitochondrial matrix acidification and hyperpolarisation which promoted ATP export. Dissipation of the mitochondrial pH gradient or pharmacological inhibition of P_i transport blocked the effects of P_i on electrochemical gradient and ATP export. Knock-down of the phosphate transporter P_iC, however, neither prevented P_i-induced mitochondrial activation nor glucose-induced insulin secretion. Using ^{31}P NMR we observed reduction of P_i pools during nutrient stimulation of INS-1E cells. Interestingly, P_i loss was less pronounced in mitochondria than in the cytosol. We conclude that matrix alkalinisation is necessary to maintain a mitochondrial P_i pool, at levels sufficient to stimulate energy metabolism in insulin-secreting cells beyond its role as a substrate for ATP synthesis.

1. Introduction

The pancreatic β-cell secretes insulin to lower blood glucose levels. In order to adjust hormone secretion to the circulating nutrient concentration, the β-cell acts as a fuel sensor. Metabolism of glucose or other nutrients inside β-cells results in the formation of signals leading to an adapted insulin secretory response (Wollheim, 2000). The best studied down-stream signal of nutrient metabolism in β-cells is the ATP/ADP ratio, which controls the electrical activity and thereby the secretion of insulin from β-cells. Glucose metabolism raises the ATP/ADP ratio, which inhibits K_{ATP} channel activity. The resulting depolarisation of the plasma membrane initiates voltage-sensitive Ca^{2+} influx the main signal for insulin granule exocytosis (Wiederkehr and Wollheim, 2006; Wollheim, 2000).

Mitochondrial oxidative phosphorylation is essential for nutrient-induced changes of the ATP/ADP ratio. Therefore mitochondria are of central importance for metabolism-secretion coupling (Wollheim, 2000). This can be demonstrated for example by inhibiting respiration and therefore mitochondrial ATP synthesis, which abolishes insulin secretion (Kennedy et al., 1998; Malaisse et al., 1979). The importance of β-cell mitochondria has also been demonstrated in animal models with impaired mitochondrial function (Silva et al., 2000) and in patients with maternally inherited diabetes due to mtDNA mutations (Maassen et al., 2005). Mitochondrial dysfunction has also been observed in β-cells of Type 2 diabetic individuals (Anello et al., 2005). Loss of mitochondrial function during disease progression may contribute to the observed blunted insulin secretion from islets of Type 2 diabetic patients (Anello et al., 2005; Maassen et al., 2005).

Mitochondrial activation during metabolism-secretion coupling can in part be explained by the nutrient-induced hyperpolarisation of the electrical potential across the inner mitochondrial membrane (Duchen et al., 1993; Wiederkehr et al., 2009). Hyperpolarisation of the electrical gradient is rapid and increases the driving force on the ATP synthase for the synthesis of ATP from ADP and inorganic phosphate (Wiederkehr et al., 2009). In addition, this hyperpolarisation promotes the export of ATP from the matrix space to the cytosol catalyzed by the adenosine nucleotide

Abbreviations: P_i, inorganic phosphate; ANT, adenine nucleotide translocator; ΔpH_{mito}, mitochondrial pH gradient; KRB, Krebs–Ringer bicarbonate; P_iC, phosphate carrier; DIC, dicarboxylate carrier; DL-αGP, DL-α-glycerol-3-phosphate; Ψ_{mito}, mitochondrial electrical potential; TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine; As_i, inorganic arsenate.

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translocator (ANT). In this transport step, ATP^{4-} is exchanged against ADP^{3-}. Hyperpolarisation of the inner mitochondrial membrane accelerates this electrogenic transport step (Klingenberg, 2008).

The second smaller component of the mitochondrial membrane potential is the chemical gradient. In general, the pH in the mitochondrial matrix is higher than in the cytosol. The resulting proton concentration gradient adds to the driving force on the ATP synthase (Wiederkehr et al., 2009). More importantly, this chemical gradient drives proton-coupled transport of metabolites or ions. Examples are the transport of inorganic phosphate or pyruvate into mitochondria, which is linked to the net uptake of protons into the matrix space (Wiederkehr and Wollheim, 2012).

Compared to other cell types, the chemical gradient in β-cells under resting conditions is very low but increases as a result of nutrient stimulation (Akhmedov et al., 2010; Wiederkehr et al., 2009). This increase of the mitochondrial matrix pH is crucial for mitochondrial activation and preventing alkalinisation blocks glucose-induced insulin secretion (Akhmedov et al., 2010). Furthermore, dissipation of the mitochondrial chemical gradient using the K^+/H^+ electro-neutral ionophore nigericin abolished substrate-induced ATP production in permeabilized cells despite the fact that the electrical gradient was enhanced (Wiederkehr et al., 2009). These results demonstrate that the mitochondrial pH gradient (ΔpH_{mito}) is a regulator of ATP generation beyond its contribution to the proton motive force. It remains to be clarified how the ΔpH_{mito} exerts its control function in mitochondrial bioenergetics.

ADP and inorganic phosphate (P_i) are the two primary substrates for ATP synthesis. Inside mitochondria, P_i may have regulatory functions controlling mitochondrial ATP synthesis in addition to its role as a substrate. For instance, P_i has been shown to increase NADH generation through the activation of Krebs cycle dehydrogenases (Hansford, 1972). P_i also augments the oxygen consumption rate possibly by facilitating the flow of electrons between cytochrome b and c (Bose et al., 2003). Cytosolic P_i enters the mitochondrial matrix together with a H^+ or in exchange for OH^- at the expense of the proton gradient (Wohlrab, 1986). P_i transport into β-cell mitochondria should therefore be sensitive to nutrient-dependent changes of the ΔpH_{mito}.

In this study we demonstrate that P_i causes a rapid and pronounced hyperpolarisation of the electrical potential across the inner mitochondrial membrane of permeabilized rat insulinoma cells. This P_i effect was linked to enhanced ATP synthesis and export from mitochondria. We further provide evidence for a net reduction of the cytosolic and mitochondrial P_i pool in intact INS-1 cells. We propose that matrix alkalinisation limits loss of the mitochondrial P_i to preserve mitochondrial activation.

2. Materials and methods

2.1. Cell culture and drugs

Rat insulinoma INS-1E cells (Merglen et al., 2004) were cultured in a humidified atmosphere (37 °C) containing 5% CO_2 in complete medium composed of RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100U/ml penicillin, and 100 μg/ml streptomycin. Experiments were performed with cells of passage number 80–120. Most chemicals were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

2.2. Permeabilisation with α-hemolysin toxin

INS-1E cells were seeded on well-plates or coverslips coated with 804G extracellular matrix which was prepared as described (Bosco et al., 2000). Cells were washed with Ca^{2+}-free Krebs-Ringer bicarbonate (KRB) solution (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 $NaHCO_3$, 2.5 mM glucose pH 7.4) and incubated in intracellular buffer (140 mM KCl, 5 mM NaCl, 7 mM $MgSO_4$, 1 mM KH_2PO_4, 20 mM HEPES, 10.2 mM EGTA, 1.65 mM $CaCl_2$, pH 7.0 titrated with KOH) with 1 μg/ml of *Staphylococus aureus* α-hemolysin toxin (Sigma) for 10 min at 37 °C (Wiederkehr et al., 2009). After α-toxin permeabilisation, cells were washed once with 0.5% BSA containing intracellular buffer and used for experiments.

2.3. ATP measurement

INS-1E cells were plated in 48 well-plates (2×10^5 cells/well), permeabilized with α-toxin and incubated for 15 min in intracellular buffer containing ADP (10 μM). After incubation, the supernatant was harvested to determine substrate and ADP dependent mitochondrial ATP release. After removal of the supernatant the cells were washed once with intracellular buffer and lysis buffer (provided by the kit) was added to extract ATP from intracellular organelles. ATP in supernatant and lysate were measured using a microplate reader (Synergy™ 2, BioTek Instruments, Inc., Winooski, VT, USA) with a bioluminescence assay kit (HS II, Roche Diagnostics; Switzerland).

2.4. Mitochondrial pH measurement

Mitochondrial matrix pH was measured using adenovirus expressing mtAlpHi a pH-sensitive fluorescent protein targeted to the mitochondrial matrix. This probe displays a close to linear fluorescence emission increase over a pH range from 7.0 to 8.5 (Abad et al., 2004). One day after plating, INS-1E cells were infected with the Ad-tetON-mtAlpHi and Ad-tetON regulatory virus for 90 min at 37 °C as described (Wiederkehr et al., 2009). After 36–48 h of culture, cells were permeabilized with α-toxin and fluorescence signals recorded using an inverted microscope (IX-81, Olympus, Tokyo, Japan) with an array laser confocal spinning disk (CSU10, Yokogawa Electric Corporation, Tokyo, Japan) and a cooled CCD camera (Cascade 512B, Photometrics, Tucson, AZ, USA). Cells were excited using 488 nm laser light and the emission wavelength was 535 nm. Images were acquired every 10 s and analysed using Metafluor 6.3 software (Universal Imaging, Molecular Devices Corporation). Titration of the mitochondrial pH was performed as described (Abad et al., 2004) by clamping the matrix pH using high K^+ buffer (125 mM KCl, 5 mM NaCl, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 10 mM HEPES) of defined pH containing the ionophores nigericin and monensin.

2.5. Mitochondrial membrane potential measurement

INS-1E cells seeded into black-walled 96 well-plates (5×10^4 -cells/well) were loaded with JC-1 (500nM, Invitrogen) for 30 min and then permeabilized with α-toxin. After washing, the cells in intracellular buffer solution, JC-1 fluorescence was monitored in a multi-well fluorescence reader (FlexStation, Molecular Devices Corporation, Sunnyvale, CA, USA). JC-1 fluorescence was measured ratiometrically: 490 nm excitation/540 nm emission (green; monomer) and 540 nm excitation/590 nm emission (red; J-aggregates) as previously described (Park et al., 2008).

2.6. Transfection with phosphate carrier (P_iC) siRNA

INS-1E cells were transfected with non-targeting or target-specific siRNA for rat P_iC using Dharmafect (Dharmacon Inc., Lafayette, CO, USA). The target-specific siRNA sequences for both P_iC-A and P_iC-B were 5′-AAAUAUGCCCUUGUACUUCUGAGGG-3′ and 5′-CCCUCAGAAGUACAAGGGCAUAUUU-3′ (Nishi et al., 2011) and siRNA