Mitochondrial oxidative stress mediates high-phosphate-induced secretory defects and apoptosis in insulin-secreting cells

Tuyet Thi Nguyen,†* Xianglan Quan,†* Kyu-Hee Hwang,† Shanhua Xu,† Ranjan Das,† Seong-Kyung Choi,† Andreas Wiederkehr,‡ Claes B. Wollheim,§ Seung-Kuy Cha,† and Kyu-Sang Park†

†Department of Physiology, Yonsei University Wonju College of Medicine, Wonju, Republic of Korea; ‡Nestlé Institute of Health Sciences, Lausanne, Switzerland, and §Department of Cell Physiology and Metabolism, University of Geneva, Geneva, Switzerland

Submitted 6 January 2015; accepted in final form 31 March 2015


Inorganic phosphate (P_i) plays an important role in cell signaling and energy metabolism. In insulin-releasing cells, P_i transport into mitochondria is essential for the generation of ATP, a signaling factor in metabolism-secretion coupling. Elevated P_i concentrations, however, can have toxic effects in various cell types. The underlying molecular mechanisms are poorly understood. Here, we have investigated the effect of P_i on secretary function and apoptosis in INS-1E clonal β-cells and rat pancreatic islets. Elevated extracellular P_i (1–5 mM) increased the mitochondrial membrane potential (ΔΨ_m), superoxide generation, caspase activation, and cell death. Depolarization of the ΔΨ_m abolished P_i-induced superoxide generation. Butylmalonate, a nonselective blocker of mitochondrial phosphate transporters, prevented ΔΨ_m hyperpolarization, superoxide generation, and cytotoxicity caused by P_i. High P_i also promoted the opening of the mitochondrial permeability transition (PT) pore, leading to apoptosis, which was also prevented by butylmalonate. The mitochondrial anti-oxidants mitoTEMPO or MnTBAP prevented P_i-triggered PT pore opening and cytotoxicity. Elevated extracellular P_i diminished ATP synthesis, cytosolic Ca^{2+} oscillations, and insulin content and secretion in INS-1E cells as well as in dispersed islet cells. These parameters were restored following preincubation with mitochondrial anti-oxidants. This treatment also prevented high-P_i-induced phosphorylation of ER stress proteins. We propose that elevated extracellular P_i causes mitochondrial oxidative stress linked to mitochondrial hyperpolarization. Such stress results in reduced insulin content and defective insulin secretion and cytotoxicity. Our data explain the decreased insulin content and secretion observed under hyperphosphatemic states.

Hyperphosphatemia; superoxide; mitochondrial permeability transition pore; insulin secretion; ER stress

INORGANIC PHOSPHATE (P_i) is essential for the most fundamental cellular functions such as signal transduction and energy metabolism (4). P_i uptake into mitochondria, for instance, is required for oxidative phosphorylation, as it is one of the essential substrates for ATP synthesis. Mitochondrial P_i also activates matrix enzymes and electron transport chain activity (5). P_i uptake into mitochondria is driven by the nutrient-generated proton gradient. In insulin-secreting cells P_i uptake also results in further hyperpolarization of the electrical gradient (36). This change accelerates the electrogenic ATP^4−/ADP^3− exchange. Exported ATP^4− acts as a main signal for insulin exocytosis by promoting closure of the plasmalemmal K_{ATP} channels (46). Consequently, mitochondrial P_i transport may contribute to the generation of signals in metabolism-secretion coupling. Consistent with such a role, P_i depletion due to low-phosphate diet impairs nutrient-stimulated insulin release (32).

Extracellular P_i is transported across the plasma membrane by types II and III Na^{+}−dependent P_i cotransporters (12). Subsequently, P_i in the cytosol is taken up into the matrix via mitochondrial P_i transporters. A number of inner membrane proteins have been proposed to contribute to such mitochondrial P_i transport: the phosphate carrier (PiC), the dicarboxylate carrier (DIC), the Mg-ATP/P_i transporter, and uncoupling protein-2 (UCP2) (7, 16, 31, 44). Physiological intracellular P_i levels in cardiac muscle detected by 31P-NMR were reported to be ~0.8 mM but elevated under ischemic or hypoxic conditions (20). In pancreatic β-cells, intracellular P_i is partially lost during nutrient stimulation, a phenomenon that is referred to as “phosphate flush” (13). Total islet P_i was also shown to be reduced during glucose-stimulated insulin secretion (42). Analysis of the cytosolic and mitochondrial P_i content in insulin-secreting cells revealed that the drop in mitochondrial P_i is lower than in the cytosol due to P_i uptake during mitochondrial activation (36). The molecular mechanism and functional implication of cellular P_i loss in response to nutrient stimulation in insulin-secreting cells remain to be investigated.

In contrast, excessive P_i load elicits cytotoxicity in a number of cell types in vitro (6, 8, 40). Serum P_i levels are maintained at about 0.8–1.5 mM in healthy individuals (10) but may increase to higher than 2 mM in some pathogenic conditions, including chronic kidney disease (47, 48). Elevated serum P_i levels can lead to cellular P_i overload, inducing osteogenic differentiation and calcification in vascular smooth muscles. A strong, positive correlation between serum P_i levels and cardiovascular morbidity and mortality has been reported (15, 38). High-P_i-induced vascular calcification was proposed to be mediated by oxidative stress (48). This is supported by data showing that P_i increases the release of reactive oxygen species (ROS) from isolated mitochondria (33).

* T. T. Nguyen and X. Quan contributed equally to this work.

Address for reprint requests and other correspondence: K.-S. Park, Dept. of Physiology, Institute of Lifestyle Medicine, Yonsei Univ. Wonju College of Medicine, Ilsan-dong, Wonju, Gangwon-Do 220-701, Republic of Korea (e-mail: q sang@yonsei.ac.kr).

http://www.ajpendo.org 0193-1849/15 Copyright © 2015 the American Physiological Society
Pancreatic β-cells display low expression of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase (41). Therefore, insulin-secreting cells are highly sensitive to oxidative stress (26), and elevated P can be particularly harmful to the pancreatic β-cells. Consistent with this possibility, in chronic kidney disease associated with hyperphosphatemia, insulin secretion is impaired (11). An epidemiologic study also shows that individuals with a higher plasma level of P have an increased risk of developing type 2 diabetes (25). Intriguingly, in “klotho”-deficient mice (ki/ki) with pronounced hyperphosphatemia (43), insulin content of the pancreas is significantly lowered compared with wild-type mice.

Appropriate provision of P, for mitochondria is indispensable for energy homeostasis and β-cell metabolism-secretion coupling. However, P overload causes oxidative stress and mitochondrial dysfunction, which may result in impaired β-cell performance and viability. In the β-cell, the phosphate flush could thus be a protective mechanism to avoid P effects. Oxidative stress as an initiator of P secretion, and cytotoxicity. We have identified mitochondrial absorbance (A540) was measured by microplate reader (Molecular Devices, Sunnyvale, CA).

Cell and drugs. Rat insulinoma INS-1E cells were cultured in humidified atmosphere (37°C) containing 5% CO2 in complete RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (HyClone; Thermo Fisher Scientific, Lafayette, CO), 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 2 mM glutamine, and 10 mM HEPES (Sigma-Aldrich, St. Louis, MO). Fluorescence dyes, including mitoSOX, TMTRM, JC-1, and calcine-AM, were purchased from Molecular Probe (Invitrogen, Eugene, OR), miTOTEMPO and Mn-TBAP were from Enzo (Enzo Life Sciences, Farmingdale, NY), and other chemicals were from Sigma-Aldrich. INS-1E cells between passages 80 and 120 were used for experiments. After plating, INS-1E cells were cultured for 24–36 h before experiments.

Pancreatic islets were isolated from 200- to 300-gram Sprague-Dawley rats ( Orient Bio, Seongnam, Korea) by collagenase (Sigma) digestion and dispersed by a brief incubation with 0.25% trypsin EDTA ( Gibco). Islet cells were seeded at a high density (3 × 10^5 cells/well) on 24-well plates coated with 804G matrix and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (45). Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Wonju Campus (YWC-100302-1).

Cell viability and apoptosis assay. Cell viability was estimated by MTT colormetric assay, which estimates the cellular reductive capacity (18). INS-1E cells were seeded on 804G-coated 96-well plates with 5 × 10^4 cells/well, and P was treated in DMEM (HyClone) without FBS for 24 h. Then, cells were incubated for 60 min at 37°C with thiazolyl blue tetrazolium bromide (Sigma-Aldrich), and the absorbance (A490) was measured by microplate reader (Molecular Devices, Sunnyvale, CA).

Apoptotic internucleosomal DNA fragmentation was quantitatively assayed by antibody-mediated capture of cytoplasmic oligonucleosome-associated histone-DNA complexes (Cell Death Detection ELISA Plus kit, Roche Diagnostics). Cells plated on 96-well plates with 5 × 10^4 cells/well were treated with P at indicated concentrations at 37°C for 24 h. Cells were suspended with 200 µl of lysis buffer supplied by the manufacturer and incubated for 30 min at room temperature. After centrifugation, 20 µl of the supernatant was used for ELISA according to the manufacturer’s protocol. Absorbance of A405 and A490 (reference wavelength), upon incubation with a peroxidase substrate for 5 min, was measured by microplate reader (Bio-Tek, Winooski, VT).

Western blot analysis. For total protein extraction, cells seeded on six-well plates were washed with ice-cold PBS and lysed with cold RIPA buffer (Thermo Fisher Scientific) containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The supernatants from lysates were loaded for SDS-PAGE and then transferred to polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA). The membrane was blocked by 5% BSA or 6% skim milk for 1 h at room temperature, followed by incubation with primary antibody at 4°C overnight. To detect the activation of caspase 3, antibody for cleaved caspase 3 (1:1,000 dilution, catalog no. 9661; Cell Signaling Technology, Danvers, MA) was used. To estimate ER stress membrane was incubated with primary antibodies for phosphorylated and total PKR-like ER kinase (PERK; 1:1,000, catalog nos. sc-32577 and sc-13073, Santa Cruz Biotechnology, Dallas, TX) or eukaryotic translation initiation factor 2α (eIF2α; 1:1,000, catalog nos. sc-101670 and sc-133132, Santa Cruz Biotechnology). β-Actin (1:5,000, catalog no. ab6267; Abcam, Cambridge, UK) was used as a loading control. Horseradish peroxidase (HRP)-conjugated secondary antibody against either mouse or rabbit IgG (catalog nos. 31450 and 31460, Thermo Scientific) was incubated for 1 h at room temperature. The bands were visualized with a UVP Biospectrum-600 imaging system using enhanced chemiluminescence (ECL) solution (Luminata Forte, Millipore, Billerica, MA).

Mitochondrial ROS and membrane potential measurements. Mitochondrial superoxide generation was determined using mitoSOX, a red fluorescence dye localized to mitochondria. Cells plated on 18-mm coverslips with 3 × 10^4 cells/well were treated with P, for the indicated time and then loaded with mitoSOX 2.5 µM for 15 min at room temperature. Fluorescence image (excitation/emission 510/580 nm) was obtained by using an inverted microscope (IX-81; Olympus, Tokyo, Japan) with confocal spinning disk (CSU10; Yokogawa, Tokyo, Japan) and analyzed by MetaMorph 6.3 software (Molecular Devices). We performed analysis from more than three independent experiments, more than 10 pictures from each, analyzing most cells in each picture.

ΔΨm was evaluated using fluorescence dyes TMRM and JC-1. Cells seeded on coverslips were loaded with TMRM (5 nM) for 10 min at 37°C and perfused at 37°C with KRB solution (in mM: 140 NaCl, 3.6 KCl, 0.5 NaH2PO4, 0.5 MgSO4, 1.5 CaCl2, 10 HEPES, 2 NaHCO3, 5.5 glucose) containing 5 nM TMRM. Real-time fluorescence imaging (excitation/emission 514/560 nm) was performed with a confocal microscopic system and analyzed by MetaFluor 6.3 software (Molecular Devices).

As an alternate potential-sensitive probe, JC-1 was used for the ΔΨm measurements in permeabilized cells. Cells seeded on black-walled 96-well plates with 5 × 10^4 cells/well were loaded with JC-1 (500 nM) for 30 min at 37°C and then permeabilized with Staphylococcus aureus α-toxin in intracellular buffer solution (in mM: 140 KCl, 5 NaCl, 7 MgSO4, 1 KH2PO4, 1.65 CaCl2, 10.2 EGTA, 20 HEPES, pH 7.0). The ratio of red (540 nm excitation and 590 nm emission) over green (490 nm excitation and 540 nm emission) fluorescence intensity was monitored from permeabilized cells with an intracellular buffer containing JC-1 (500 nM) using a multi-well fluorescence reader (FlexStation II, Molecular Devices) as described (37). JC-1 was also used for detecting the P-induced long-term (<6 h) changes in ΔΨm in intact cells. Cells in the same 96-well plate were loaded with JC-1 (300 nM) for 30 min. After a washing, the changes in JC-1 fluorescence ratio with P, treatment were measured using the multi-well fluorescence reader (FlexStation II, Molecular Devices).

Measurement of mitochondrial permeability transition (PT) pore opening. The opening of mitochondrial PT pore in intact cells can be traced directly by changes in calcine fluorescence, as described previously (14, 34). Briefly, cells seeded on black-walled 96-well plates (5 × 10^4 cells/well) were pretreated with or without P, for the
indicated time and loaded with calcine-AM (1 μM) for 30 min at room temperature in the dark. Calcine-AM can diffuse freely into cellular organelles including mitochondria and be trapped after cleavage by esterase. Subsequently, after washout, cells were incubated with CoCl$_2$ (1 mM) for a further 15 min at room temperature. Cobalt ion (Co$^{2+}$) quenches calcine fluorescence in all subcellular compartments except the mitochondrial matrix. Cells were washed again, and fluorescence intensity from calcine (excitation/emission 485/530 nm) was monitored with the multi-well fluorescence reader (Flexstation II). During the opening of PT pore, Co$^{2+}$ enters mitochondria and is able to quench mitochondrial calcine fluorescence.

**Intracellular Ca$^{2+}$ measurement.** Cells seeded onto 12-mm cover-slips (3 × 10$^4$ cells) and were treated with 3 mM P$_i$ for 2.5 h in 2.8 mM glucose-KRB solution at 37°C. After washout, cells were incubated with 2.8 or 16.7 mM glucose for 10 min at 37°C and lysed with a buffer supplied with the ATP bioluminescence assay kit (HSI, Roche Diagnostic). Lysates were harvested, and ATP level was measured by microplate luminometer (Synergy 2, Bio-Tek Instruments) with the assay kit.

To measure SO$_4^-$ content and secretion, cells seeded on 24-well plates were treated with 3 mM P$_i$ for 2.5 h in 2.8 mM glucose-KRB solution. The cells were then incubated for 30 min at 37°C with 2.8 or 16.7 mM glucose-KRB solution containing 0.1% bovine serum albumin (the different P$_i$ concentrations were maintained). The supernatant and the cell extracts by acid-ethanol were collected, and the levels of insulin release and content were measured following the manufacturer's protocol with a rat insulin ELISA kit (Shibayagi, Gunma, Japan). The absorbance (A$_{450}$) was measured in a microplate reader (AJP-Endocrinol Metab) at 37°C. After washout, cells were incubated with 2.8 or 16.7 mM glucose for 10 min at 37°C and lysed with a buffer supplied with the ATP bioluminescence assay kit (HSI, Roche Diagnostic). Lysates were harvested, and ATP level was measured by microplate luminometer (Synergy 2, Bio-Tek Instruments) with the assay kit.

**ATP and insulin measurements.** For measurement of ATP content, cells seeded on 24-well plates (2 × 10$^5$ cells/well) were treated with 3 mM P$_i$, then incubated for 1 h, demonstrating facilitation of oxidative stress and apoptosis.
of Ca^{2+}-triggered PT pore opening by P_{i} (Fig. 2, E and F). Longer incubations with P_{i} (3 mM for 3 h) reduced calcine fluorescence due to direct effect of P_{i} on PT pore opening, which was prevented by BMA (Fig. 2, G and H). We also observed depolarization of the ΔΨ_{m} by long-term incubation with P_{i} (1–3 mM for 6 h) as monitored using the JC-1 fluorescence dye (JC-1 ratio: 3.48 ± 0.31 at 1 mM P_{i}, 1.07 ± 0.09 at 3 mM P_{i}, P < 0.001).

Oxidative stress-mediated secretory defects and apoptosis. ATP, generated by oxidative phosphorylation, is a main signal in β-cell metabolism-secretion coupling, regulating plasma membrane depolarization and thereby [Ca^{2+}], influx (46). As shown in Fig. 3A, ATP production in response to 10 min of glucose stimulation was reduced by preincubation with high P_{i} for 3 h (Δincrease: 1.5 ± 2.2 nmol/mg protein at 3 mM, 7.7 ± 1.9 at 1 mM, P < 0.05), consistent with the observed PT pore opening and depolarization of the ΔΨ_{m}. Glucose-stimulated [Ca^{2+}], oscillations were also markedly reduced by P_{i} exposure (3 mM for 3 h; Fig. 3, B and C). The impact of high P_{i} on metabolism-secretion coupling was also analyzed in primary pancreatic cells. Dispersed rat islet cells showed a more than threefold increase in insulin release upon stimulation by high glucose incubation (16.7 mM for 30 min). Glucose-stimulated insulin secretion was significantly impaired in islet cells following exposure to 3 mM P_{i} for 3 h (Fig. 3D). Cellular insulin content also showed a decrease in high-P_{i}-incubated islet cells (Fig. 3E). It has been suggested that oxidative stress elicits ER stress, including the unfolded protein response (UPR), which plays pathogenic roles in type 2 diabetes (2). We observed phosphorylation of PKR-like ER kinase (PERK) and eukaryotic translation initiation factor 2α (eIF2α), two markers of the UPR after 1-h exposure to 3 mM P_{i} in dispersed islet cells (Fig. 3F).

To demonstrate the role of oxidative stress in defective insulin secretion, we used the mitochondrial antioxidants mitoTEMPO or Mn-TBAP. These antioxidants successfully scavenged mitochondrial superoxide triggered by high P_{i} (Fig. 4A). Phosphorylation of PERK and eIF2α by high P_{i} was completely blocked by preincubation with the mitochondrial antioxidant mitoTEMPO both in rat pancreatic islet cells and INS-1E cells (Fig. 4, B–D). Glucose-stimulated insulin secretion and total cellular insulin content in INS-1E cells were reduced by high P_{i} (3 mM for 3 h). These conditions did neither alter the cell number nor total cellular protein (data not shown). Impaired insulin release and decreased cellular content were restored by the pretreatment with mitoTEMPO (100 nM; Fig. 4, E and F). Mitochondrial antioxidants prevented P_{i}-triggered PT pore opening (3 mM P_{i} for 3 h; Fig. 4G) and cytotoxicity (3 mM P_{i} for 24 h; Fig. 4H). These results demonstrate that superoxide generation induced by mitochondrial P_{i} uptake and ΔΨ_{m} hyperpolarization contribute not only to the lowering of

Fig. 1. High extracellular phosphate elicits mitochondrial hyperpolarization, superoxide generation, and apoptotic cell death in INS-1E cells. INS-1E cells were treated with P_{i} at different concentrations for 24 h (A) and with 3 mM for different incubation times (B). P_{i}-induced cytotoxicity was assessed by MTT assay. In each P_{i}-treated group, equimolar NaCl was used as control. Western blot and densitometric analysis for cleaved caspase 3 (C) and ELISA for apoptotic DNA fragments (D) were performed in P_{i} (24-h)-treated cells. Mitochondrial superoxide production and membrane potential (ΔΨ_{m}) were measured using a confocal microscope with the mitochondrial ROS indicator mitoSOX (E) and the potential sensitive dye TMRM (F and G), respectively. G: changes in TMRM intensity by P_{i} incubation (30 min) were normalized to the basal fluorescence level. H: effects of FCCP (0.3–1 μM) on mitochondrial superoxide generation by high P_{i} (60 min) were analyzed. *P < 0.05, ***P < 0.001.
releasable insulin content but also to PT pore opening, mitochondrial dysfunction, and apoptosis.

**DISCUSSION**

Phosphorus homeostasis is maintained by balancing intake from diet, storage, and loss from the body via renal/fecal excretion (38). Increases in serum P_i concentrations lead to decreased gastrointestinal absorption or renal tubular reabsorption by closely modulating the hormonal control, mainly by PTH, of vitamin D and fibroblast growth factor (FGF)23 (4, 15). Uncompensated hyperphosphatemia induced by chronic kidney disease or inappropriate hormonal control may cause medial calcification or Monckeberg’s sclerosis due to transdifferentiation of vascular smooth muscle cells into osteogenic precursors (38). Elevated P_i (remaining within the normal physiological range) positively correlates with the development of cardiovascular complications and mortality (15). It is noteworthy that mice lacking the klotho protein, a coreceptor for FGF23, show accelerated aging and depletion of pancreatic insulin (38, 43). The phenotype of these mice was alleviated by providing a low P_i diet, demonstrating the direct pathogenic role of high P_i (30). Defective insulin secretion is also observed in uremia-associated hyperphosphatemia; however, the relationship between phosphate toxicity and impaired β-cell function has not been clearly established (11, 27). The present study provides evidence that high P_i exposure triggers PT pore opening, mitochondrial dysfunction, and apoptosis, all of which result from mitochondrial P_i uptake and superoxide production. In rat clonal β-cells as well as pancreatic islets, high P_i decreases insulin content and impairs nutrient-stimulated insulin release due to mitochondrial ROS formation. These results underline the pathophysiological role of mitochondrial oxidative stress as mediator of phosphate toxicity in insulin-secreting cells, which have a very dense mitochondrial network but a rather weak antioxidant capacity (41).

We (36) previously reported that mitochondrial P_i uptake is driven by the pH gradient. P_i uptake therefore dissipates the chemical gradient but at the same time elevates the electrical gradient under the maintained proton motive force. P_i also facilitates oxidative phosphorylation due to activation of metabolic enzymes and respiratory chain activity, which can further elevate the electrical gradient (5). In this study, we...
observed hyperpolarization of the $\Delta \Psi_m$ following acute addition of $P_i$ to intact as well as permeabilized cells (Figs. 1F and 2A). Mitochondrial superoxide production is also accelerated relatively early during $P_i$ incubation, coincident with the kinetics of $\Delta \Psi_m$ hyperpolarization. In addition, $P_i$-induced ROS formation was prevented by a mitochondrial uncoupler that collapses the established electrochemical gradient. These results suggest that $P_i$-induced ROS generation is linked to the increased $\Delta \Psi_m$ occurring as a result of mitochondrial $P_i$ uptake. This is in agreement with previous reports showing that

---

**Fig. 3.** High phosphate impairs insulin secretion and initiates UPR in rat pancreatic islet cells and INS-1E cells. Glucose (16.7 mM)-stimulated ATP synthesis (A) and intracellular $Ca^{2+}$ oscillation (B–C) were measured in INS-1E cells incubated with either 1 or 3 mM $P_i$ (3 h). Dispersed rat pancreatic islets were plated at high density. Insulin release and content were measured after glucose stimulation (30 min) following preincubation with $P_i$, for 3 h (D & E). Phosphorylation of PKR-like ER kinase (PERK) and eukaryotic translation initiation factor 2a (eIF2a) in islet cells incubated with different $P_i$ concentration (1–5 mM for 1 h) were analyzed (F). *$P < 0.05$.

**Fig. 4.** Mitochondrial antioxidants block high-phosphate-induced secretory defects, ER stress, permeability transition, and cell death. Mitochondrial antioxidant mitoTEMPO (mTP) or Mn-TBAP (MnTB) was added 30 min before $P_i$ incubation. Effects of mitochondrial antioxidants on $P_i$-induced mitochondrial superoxide generation (A) ($P_i$ for 1 h), phosphorylation of PKR-like ER kinase (PERK), and eukaryotic translation initiation factor 2a (eIF2a) in rat pancreatic islets (B) and INS-1E cells (C and D) ($P_i$ for 1 h), insulin release and content (E and F) ($P_i$ for 3 h), permeability transition (PT) (G) ($P_i$ for 3 h), as well as cytotoxicity (H) ($P_i$ for 24 h). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 

E938 HIGH $P_i$ IN INSULIN-SECRETING CELLS

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00009.2015 • www.ajpendo.org
elevated $\Delta \Psi_m$ favors electron transport chain-dependent ROS synthesis (19).

Several transporters of the SLC25 family, localized in the inner mitochondrial membrane, have been suggested to mediate $P_i$ uptake into the organelle. For example, the phosphate carrier (PiC) is a member of the SLC25 family (SLC25A3) and has two isoforms, PiC-A and PiC-B. PiC-A is expressed in skeletal and cardiac muscle, whereas PiC-B is expressed ubiquitously (9). Patients with PiC-A deficiency show muscle weakness with lactic acidosis, demonstrating the physiological importance of this transporter (28). In insulin-secreting cells, the functional role of PiC remains controversial (31, 36). In our previous work (36), we did not observe any significant alterations in $P_i$-induced $\Delta \Psi_m$ hyperpolarization and ATP generation in INS-1E cells when knocking-down both PiC isoforms. Alternative $P_i$ transport mechanisms must exist. DIC, for instance, has been proposed as a functional mitochondrial $P_i$ transporter in pancreatic $\beta$-cells. Depletion of DIC impairs nutrient-stimulated insulin secretion (17). Recently, UCP2 was reported to transport $P_i$ into mitochondria with or in exchange for carbohydrate metabolites (44). In our hands, knockdown of neither DIC nor UCP2 influences $P_i$-induced changes. To block $P_i$ uptake, we therefore used the nonselective blockers BMA or mersalyl, known to inhibit all the above-listed $P_i$ transporters (17, 36, 44). Pretreatment with BMA abrogated $\Delta \Psi_m$ hyperpolarization, superoxide generation, and cytotoxicity induced by high $P_i$, emphasizing the importance of mitochondrial $P_i$ transporters in this pathogenic process.

The mitochondrial PT pore was proposed to be a multiprotein complex composed of mitochondrial inner and outer membrane proteins such as porin, adenine nucleotide translocase (ANT), and cyclophilin D (CyP-D) (49). Of note, the pore-forming component of PT was suggested to be PiC, which may be converted into a nonselective large-conductance channel when bound to ANT or CyP-D (23). Cyclosporin A (CsA) inhibits PT by binding to CyP-D, which depends on $P_i$ transporters (3, 17). The properties of PT vary depending on the tissue; for instance, the sensitivity to CsA is very low in mitochondria from brain or pancreatic $\beta$-cells (21). The factors regulating PT pore opening include oxidative stress, adenine nucleotide depletion, $pH$, and mitochondrial matrix $Ca^{2+}$ or $P_i$ overload. These stimuli cooperate to increase the permeability of the inner mitochondrial membrane resulting in the loss of $\Delta \Psi_m$, mitochondrial swelling, and rupture of the outer mitochondrial membrane, causing release of proapoptotic intermembrane proteins into the cytosol. Using the calcine-CoCl$_2$ technique, we observed increased permeability to CoCl$_2$ via PT pore opening induced by ionomycin-dependent $Ca^{2+}$ overload. $Ca^{2+}$-triggered PT pore opening was accelerated following a 1-h preexposure to high $P_i$ (Fig. 2, $E$ and $F$). $P_i$ incubation for 3 h alone (without $Ca^{2+}$ stress) also caused PT, which was abolished by BMA (Fig. 2, $G$ and $H$). This evidence demonstrates that $P_i$-induced PT pore opening requires mitochondrial $P_i$ uptake. High mitochondrial $P_i$ concentration and oxidative stress may work additively or synergistically to facilitate this process. Our work reveals that PT triggered by long-term incubation with high $P_i$ causes depolarization of the $\Delta \Psi_m$. Mitochondrial depolarization is observed at a late stage, preceded by apoptotic cell death induced by the elevated $P_i$ concentration. Mitochondrial dysfunction due to PT may be responsible for $P_i$-induced defects in glucose-stimulated ATP synthesis, $[Ca^{2+}]_i$ oscillations, and insulin secretion.

Insulin secretion from pancreatic $\beta$-cells is sensitive to ROS in either a positive or a negative manner. Regulated ROS production at relatively low concentrations may act as a positive signal in metabolism-secretion coupling (35). On the other hand, sustained and uncontrolled oxidative stress has detrimental effects on mitochondrial function and insulin secretion (26). It is noteworthy that even brief oxidative stress exerts persistent defects in mitochondrial function and glucose-stimulated insulin secretion (24). The present study demonstrates that $P_i$-induced mitochondrial superoxide formation has noxious effects on cellular insulin content and secretion at early time points before cytotoxic changes are observed. Defective glucose-stimulated insulin secretion could result from depletion of insulin content. However, the amount of insulin release is less than 1% of total insulin content even in the presence of high $P_i$. Instead, we propose that mitochondrial dysfunction via PT pore opening could account for impaired glucose-stimulated ATP synthesis, $Ca^{2+}$ oscillation, and insulin release. ER homeostasis is important for $\beta$-cell function, especially given the necessity to synthesize the large amounts of insulin required to maintain glucose homeostasis. It has been proposed that insulin synthesis in response to high glucose stimulation poses a burden on the ER protein folding machinery, leading to ER stress. To alleviate the accumulation of unfolded or misfolded proteins, translation of proteins including insulin is attenuated as a result of the UPR. Particularly, pancreatic $\beta$-cells have an extremely high protein folding load and an increased tendency to have ER stress upon oxidative stress (39). Because of the close interaction between mitochondria and the ER, mitochondrial ROS can disrupt ER redox homeostasis and elicit ER stress due to ER $Ca^{2+}$ depletion (1). Strengthening the antioxidant system in $\beta$-cells has been demonstrated to improve ER protein folding capacity and insulin biosynthesis (29). In the present study, we have demonstrated that mitochondrial oxidative stress by high $P_i$ triggers phosphorylations of PERK and eIF2$\alpha$, which leads to attenuation of protein translation. Prevention of mitochondrial superoxide generation restored insulin content and glucose-stimulated insulin secretion due to improved ER function as well as mitochondrial activity. We propose two distinct mechanisms for insulin secretion defects and diminution of insulin content. The former is more related to mitochondrial dysfunction, and the latter is the outcome of UPR initiated by $P_i$-generated ROS. This provides strong evidence for the critical role of mitochondrial ROS in the pathogenic consequences induced by high $P_i$ in pancreatic $\beta$-cells.

$P_i$ retention plays a causative role in endothelial dysfunction and vascular calcification in uremic patients. Even individuals with elevated serum levels of $P_i$ within the “normal” range have a significantly increased risk of cardiovascular and metabolic diseases, as reported in a number of independent studies (10, 25). We suggest that long-term exposure to moderate elevation of $P_i$ induces chronic deterioration of insulin secretion. Intriguingly, when different mammals are compared, an inverse correlation between longevity and serum $P_i$ concentrations has been observed, but little is known about the mode of action of $P_i$ in the aging process (22). Here, we demonstrate that oxidative stress related to mitochondrial $P_i$ uptake is responsible for defective insulin synthesis and secretion. This may be an important mechanism adding to the complications observed as a result of hyperphosphatemia and contribute to
accelerate the ageing process. Inhibition of mitochondrial P1 transporters and the activation of the mitochondrial antioxidant system may be therapeutic approaches to treat phosphate toxicity with the potential to also improve age-related metabolic disease.

GRANTS

This study was supported by a grant from the Myung Sun Kim Memorial Foundation (2014).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES