Original article

Genetic manipulation of the cardiac mitochondrial phosphate carrier does not affect permeability transition

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ABSTRACT

The Mitochondrial Permeability Transition (MPT) pore is a voltage-sensitive unselective channel known to instigate necrotic cell death during cardiac disease. Recent models suggest that the isomerase cyclophilin D (CypD) regulates the MPT pore by binding to either the F_0F_1-ATP synthase lateral stalk or the mitochondrial phosphate carrier (PiC). Here we confirm that CypD, through its N-terminus, can directly bind PiC. We then generated cardiac-specific mouse strains overexpressing or with decreased levels of mitochondrial PiC to assess the functionality of such interaction. While PiC overexpression had no observable pathologic phenotype, PiC knockdown resulted in cardiac hypertrophy along with decreased ATP levels. Mitochondria isolated from the hearts of these mouse lines and their respective non-transgenic controls had no divergent phenotype in terms of oxygen consumption and Ca^{2+}-induced MPT, as assessed by swelling and Ca^{2+}-retention measurements. These results provide genetic evidence indicating that the mitochondrial PiC is not a critical component of the MPT pore.

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1. Introduction

In cardiomyocytes, mitochondria are responsible for key metabolic pathways that will determine the fate of the cell. The normal activity of these organelles is essential for processes such as fatty acid oxidation, porphyrin synthesis, ion homeostasis, and oxidative phosphorylation. However, mitochondrial dysfunction is seldom compatible with cellular viability and is often a cause of myocardial pathologies. Conditions threatening cardiac mitochondrial homeostasis such as ischemia/reperfusion injury [1], diabetes [2], or the damage induced by chemotherapeutics [3] are often associated with increases in unselective mitochondrial permeability to water, ions and metabolites. These changes in permeability induce mitochondrial respiratory chain collapse, oxidative stress, and ultimately swelling and rupture of the organelle, thereby triggering death of the cardiomyocyte [4].

Opening of the MPT pore, a non-selective channel thought to span the inner mitochondrial membrane, is known to be the underlying cause of the permeability changes that initiate mitochondrial-induced cell death [5]. For over 2 decades, the MPT pore was thought to be the consequence of a redox-induced conformational change of the adenine nucleotide translocase (ANT) in the inner membrane catalyzed by the chaperone cyclophilin-D (CypD) in the matrix [6]. However, studies in gene-knockout mice have cast doubt on the validity of this model as mice lacking ANT or CypD still exhibit an MPT response, albeit with diminished sensitivity to ANT and CypD ligands respectively [7–10]. The Ca^{2+}-induced MPT pore is effectively modulated by accessory CypD overexpression or gene-targeting [9]. Thus ANT and CypD appear to be regulators of the MPT pore rather than the pore itself. More recent hypotheses propose that the F_1F_0-ATP synthase or the mitochondrial phosphate carrier (PiC) may instead form the MPT pore, where binding of these proteins to CypD is affected by CsA [11–15]. However, both of these models still need extensive testing and genetic validation as CypD may bind to other proteins such as Hsp90/TRAP1, Bcl-2 and C1qbp [16–18].

Here, we investigated the role of PiC in comprising the MPT pore through structural, genetic and bioenergetic approaches to further understand the role of this unselective mitochondrial channel in cardiac

cell death and the progression of cardiovascular disease. We confirm that PiC binds to CypD and map the interaction site to the N-terminus of CypD. To assess the functionality of such an interaction, we generated cardiac-specific transgenic mouse strains overexpressing PiC from 4 to 6 fold and a PiC knockdown strain where the carrier expression was decreased ~60%. PiC overexpression, either alone or in conjunction with CypD upregulation, resulted in no obvious phenotypic pathology per se. PiC knockdown resulted in cardiac hypertrophy with decreased fractional shortening thus partially recapitulating the outcome of PiC deficiency observed in humans [19,20]. Isolated cardiac mitochondria from the lines either overexpressing or underexpressing PiC levels still displayed a typical Ca^{2+}-induced MPT pore response with comparable characteristics. Our results suggest that the level of expression of PiC in mouse mitochondria has little effect on classical readouts of the MPT pore opening. This argues against a main role of PiC as the MPT pore-forming component.

2. Experimental procedures

2.1. Animals

All the experiments involving mice were approved by the University of Missouri Animal Care and Use Committee and conformed to the NIH guidelines for the use and care of animals. To generate transgenic (TG) mice, mouse PiC cDNA was inserted in the α-myosin heavy chain (MHC) promoter cassette and injected into fertilized FVB/N oocytes. PiC shRNA TG mice were generated as described previously [21]. Briefly, a shRNA against mouse PiC (Open Biosystems) was subcloned downstream of a tetracycline responsive minimal CMV promoter in the pTMP vector (Open Biosystems). The transgene was linearized with BglII and AgeI and injected into fertilized FVB/N oocytes. The resultant mice were then crossed with cardiac-specific tTA driver mice [22] to express the shRNA, the target sequence of which was 5′-tcaacaagcagattcagtc-3′. All experiments were performed when the mice were 2 months of age and comparisons were made against respective non-transgenic (NTG) littermates.

2.2. Echocardiography

Echocardiograms were performed using a GE Vivid 1 ultrasound system with a 12-mHz transducer. Analysis was performed offline using GE EchoPAC Software.

2.3. Mitochondrial isolation and assays

Heart mitochondria were prepared from the different mouse lines by differential centrifugation in sucrose-based medium, as previously described [9,23]. Mitochondria were resuspended in swelling buffer (120 mM KCl, 10 mM Tris at pH 7.4 and 5 mM KH_2PO_4) at 0.20 mg/mL. MPT was measured spectrophotometrically as a decrease in light scattering at 520 nm and was induced by the addition of 250 μM $CaCl_2$ to de-energized mitochondria. In calcium retention capacity (CRC) experiments, extramitochondrial Ca^{2+} was measured fluorimetrically using 1 μM Calcium Green-5N. Mitochondria were resuspended in swelling buffer supplemented with 10 mM succinate to a concentration of 0.125 mg/mL in a final volume of 1 mL. 1 μL from a stock solution of 2.5 mM $CaCl_2$ was added each minute until an increase in fluorescence due to Ca^{2+}-release was detected. Oxygen consumption was measured polarographically at 25 °C using a Clark-type electrode in the medium used for swelling measurements supplemented with 1 mM $MgCl_2$ and either 5 mM glutamate/5 mM malate or 10 mM succinate. State 3 was initiated by adding 200 μM ADP to the reaction mixture. ATP levels from mitochondrial fractions were determined using the CellTiter-Glo luminescence assay from Promega according to the manufacturer's instructions.

2.4. Adult mouse cardiomyocyte isolation and evaluation of CRC

Mice were anesthetized with pentobarbital sodium (60 mg/kg) and after deep anesthesia was confirmed the chest cavity was opened and the heart were rapidly (~30s) removed and placed in 4 °C Ca^{2+}-free physiological saline solution (PSS, 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 10 mM Hepes, pH 7.4). Excised hearts were immediately cannulated via the aorta and retrogradely perfused with Ca^{2+}-free PSS containing 2U/mL heparin for 10 min, followed by 15–16 minute perfusion with a minimal essential medium (MEM)-based enzymatic isolation solution supplemented with: 10 mM $NaHCO_3$, 2 mM Na-Pyruvate, 10 mM NaHEPES, 10 mM HEPES, 8 mM taurine, 20 μM $CaCl_2$, 50,000 U/L PenStrep, and 22.5 μg/mL Liberase Blendzyme TH (Roche Applied Science), pH 7.35. Hearts were removed from the perfusion unit and placed in a MEM-based solution supplemented with 10 mM $NaHCO_3$, 2 mM Na-Pyruvate, 10 mM NaHEPES, 10 mM HEPES, 40 μM $CaCl_2$, 50,000 U/L PenStrep, and 10 mg/mL bovine serum albumin. The left-ventricle and septum were minced, agitated, and filtered (200 μm nylon mesh) to obtain isolated cardiomyocytes. Cardiomyocytes were counted (30,000 per experiment) and resuspended with 1 mL of buffer containing 120 mM KCl, 10 mM Tris at pH 7.4, 1 mM KH_2PO_4, 10 mM succinate, 20 μM EDTA and 0.5 μg digitonin. CRC was measured as described for isolated mitochondria by detecting changes in the fluorescence of 1 μM Calcium Green-5N at 530 nm.

2.5. Western blotting

Cardiac whole tissue and mitochondria were homogenized in buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, and 1% Triton-X100. Proteins were resolved by SDS-PAGE using 10% acrylamide, transferred onto PVDF membranes, and immunoblotted using the following commercially available antibodies: anti-ATP synthase, anti-CypD, anti-GST, and anti-VDAC from Abcam; and anti-ANT from Santa Cruz. The polyclonal PiC antibody was custom made for us by YenZym. Membranes were then incubated with the appropriate alkaline phosphatase-linked secondary antibody (Cell Signaling) and visualized by enhanced chemifluorescence (Amersham).

2.6. Pulldowns

Mature CypD and the various CypD truncation mutants were amplified by PCR and subcloned into the pGEX-4T1 vector. The resultant recombinant proteins were then incubated with cardiac mitochondrial lysates plus glutathione sepharose beads overnight at 4 °C. After washing 3 times with homogenization buffer the complexes were then subjected to Western blotting for PiC and GST. For the direct interaction studies, His-tagged recombinant CypD (ProSpec) was incubated with GST-PiC (Abnova) for 15 min at RT in PBS plus 0.1% NP-40, and the resultant complexes purified using Co^{2+} affinity columns or glutathione sepharose.

2.7. Protein modeling and docking

The murine mitochondrial PiC model was generated on I-TASSER [24] using the mitochondrial ADP/ATP carrier PDB model 1OKC as template. The available CypD PDB model 2BIT was used for further docking simulations with the PiC model using RosettaDock [25]. The starting conformation for local docking search was set by placing CypD near the PiC domains corresponding to the mitochondrial matrix portion of the carrier. All output models were considered for further analysis and were rendered using PyMol [26].

2.8. Statistical analyses

Data are presented as mean ± s.e.m. Statistical evaluation between 2 groups was performed by unpaired t-tests. For 3 groups, one-way