Biochimica et Biophysica Acta 1817 (2012) 1860–1866

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Induction of the permeability transition pore in cells depleted of mitochondrial DNA☆

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ARTICLE INFO

Article history:
Received 20 January 2012
Received in revised form 16 February 2012
Accepted 21 February 2012
Available online 28 February 2012

Keywords:
Mitochondrion
ρ^0 cell
Permeability transition
Cyclophilin D
Hexokinase
GSK3β

ABSTRACT

Respiratory complexes are believed to play a role in the function of the mitochondrial permeability transition pore (PTP), whose dysregulation affects the process of cell death and is involved in a variety of diseases, including cancer and degenerative disorders. We investigated here the PTP in cells devoid of mitochondrial DNA (ρ^0 cells), which lack respiration and constitute a model for the analysis of mitochondrial involvement in several pathological conditions. We observed that mitochondria of ρ^0 cells maintain a membrane potential and that this is readily dissipated after displacement of hexokinase (HK) II from the mitochondrial surface by treatment with either the drug clotrimazole or with a cell-permeant HK II peptide, or by placing ρ^0 cells in a medium without serum and glucose. The PTP inhibitor cyclosporin A (CsA) could decrease the mitochondrial depolarization induced by either HK II displacement or by nutrient depletion. We also found that a fraction of the kinases ERK1/2 and GSK3α/β is located in the mitochondrial matrix of ρ^0 cells, and that glucose and serum deprivation caused concomitant ERK1/2 inhibition and GSK3α/β activation with the ensuing phosphorylation of cyclophilin D, the mitochondrial target of CsA. GSK3α/β inhibition with indirubin-3′-oxime decreased PTP-induced cell death in ρ^0 cells following nutrient ablation. These findings indicate that ρ^0 cells are equipped with a functioning PTP, whose regulatory mechanisms are similar to those observed in cancer cells, and suggest that escape from PTP opening is a survival factor in this model of mitochondrial diseases. This article is part of a Special Issue entitled: 17th European Bioenergetics Conference (EBEC 2012).

1. Introduction

The creation of cells depleted of mitochondrial DNA (mtDNA), termed ρ^0 cells [1,2], has been a major advance in the study and understanding of mtDNA diseases. The availability of ρ^0 cells allowed their repopulation with different mitochondrial genomes, and thus provided an essential tool to define key pathogenic parameters such as the degree of heteroplasmy and threshold levels of mutant mtDNA [3,4]. ρ^0 cells represent an extreme case of mitochondrial dysfunction that has also attracted considerable interest in the fields of bioenergetics and cell death [5–18], in particular about the intrinsic pathway to apoptosis that is triggered by release of mitochondrial apoptogenic proteins [19] and about the effector mechanisms of necrosis caused by bioenergetic failure [20].

One candidate mechanism for the irreversible commitment to cell death is the mitochondrial permeability transition pore (PTP), an inner membrane cyclosporin (Cs) A-sensitive, high-conductance channel whose opening causes mitochondrial depolarization and a variety of effects that depend on the PTP open time [21]. Whereas short open times may take part in physiological Ca^{2+} signalling by providing mitochondria with a fast Ca^{2+} release channel [22–26], longer openings lead to rearrangement of the cristae and to matrix swelling and eventually prompt outer membrane rupture followed by release of cytochrome *c* and of other proapoptotic proteins (such as AIF, Smac/DIABLO and endonuclease G) for which there is no selective release pathway in the outer membrane [27].

Despite the crucial importance of PTP in cell biology and in the pathogenesis of a number of diseases [28], its molecular composition remains an unsolved riddle. The activity of RC complexes and of ATP synthase can influence the PTP, as ROS generated as a respiratory by-product can elicit pore openings [29]. We have demonstrated that the PTP modulator cyclophilin D (CyP-D) binds and inhibits the ATP synthase [30] and that the activity of RC complex I contributes to setting the threshold voltage for PTP opening [31], providing molecular and conceptual bases for the existence of a functional interplay between

Abbreviations: Cs, cyclosporin; CyP-D, cyclophilin D; $\Delta\psi_m$, mitochondrial membrane potential difference; EGTA, ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; HK II, Hexokinase II; PTP, permeability transition pore; TAT-HK II, KMIASHLLAYFFTELNβA-GYGRKKRRQRRRG peptide; RC, respiratory chain; TMRM, tetramethylrhodamine methyl ester

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doi:10.1016/j.bbabio.2012.02.022

respiration and the PTP. Moreover, we have recently shown that PTP opening plays a pathogenic role in two models of mitochondrial diseases endowed with inactivation of RC complex I, *i.e.* HL180 cybrids harboring two missense mutations causative of Leber Hereditary Optic Neuropathy in the mtDNA ND6 gene [32,33], and the cell line XTC.UC1 (derived from a thyroid oncocytoma [34]) that bears a disruptive frameshift mutation in the mtDNA ND1 gene [35]. Nonetheless, whether the PTP is involved in other mtDNA disease models is not known, and a formal definition of the role played by RC complexes in PTP regulation and molecular composition is still lacking.

Cells devoid of mtDNA also constitute a potential model for the study of the bioenergetics of neoplasias, where mitochondrial respiration is decreased and ATP is largely provided by inducing glycolysis and uncoupling it from oxygen availability, the Warburg effect [36–38]. Thus, ρ^0 cells can be considered as possessing an extreme "Warburg phenotype". Furthermore, reduction of mtDNA copy number was reported in diverse types of tumors such as stomach, colon, lung [39], hepatocellular [40], ovarian [41] and breast carcinomas [42] and astrocytomas [43]; mtDNA was also reported to affect angiogenesis, invasiveness [44,45] and resistance to death of tumor cells [15,46]. We and others have observed that mitochondria from tumor cells display an increased resistance to PTP opening as a death-escaping strategy, and this is achieved both by deploying hexokinase (HK) II on the mitochondrial surface, where it maintains the PTP in a closed state through a poorly defined signal transduction cascade directed to matrix components such as CyP-D [47]; and by constitutively inducing an ERK/GSK3 signalling pathway that maintains CyP-D in a dephosphorylated state, thus enhancing the threshold for PTP induction [48,49].

In keeping with these data, here we observe that in human osteosarcoma 206 ρ^0 cells the PTP is induced both by HK II detachment from mitochondria, and by modulation of an ERK/GSK3/CyP-D transduction axis following glucose and serum depletion.

2. Materials and methods

2.1. Materials, cell cultures and growth conditions

Tetramethylrhodamine methyl ester (TMRM) was from Molecular Probes (Eugene, OR); FITC-conjugated annexin-V was from Boehringer Mannheim (Indianapolis, IN); indirubin-3′-oxime and CsA were from Calbiochem (San Diego, CA); cyclosporin H (CsH) was a generous gift of Dr. Urs Ruegg, Geneva; all other chemicals were from Sigma (Milan, Italy). Mouse monoclonal anti GSK-3α/β, Grim19, SDHA and UQCRC1, goat polyclonal anti calnexin, HK II and VDAC1, and rabbit polyclonal anti ERK2, PARP and TOM20 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal anti phospho-ERK1/2 (Thr202/Tyr204) and anti phospho GSK-3α/β (Ser21/9) and rabbit monoclonal anti caspase-3 antibodies were from Cell Signaling (Beverly, MA); mouse monoclonal anti phospho-Ser/Thr, CyP-D and anti prohibitin antibodies were from Qiagen, Calbiochem, and Lab Vision (Fremont, CA), respectively; rabbit polyclonal anti AIF antibody was from Exalpha Biologicals (Shirley, MA); the mouse monoclonal OXPHOS antibody cocktail recognizing CI (NDUFB8), CII (SDHB), CIII (core2), CIV (COXII) and CV (α) was from Mito-Sciences (Eugene, OR). Peptides MIASHLLAYFFTELNβA-GYGRKKRRQRRRG (TAT-HK II) and GYGRKKRRQRRRG-βA-EEEAKNAAAKLAVEILNKEKK (TAT-Ctrl) were synthesized by a solid phase method using an automatized peptide synthesizer (model 431-A, Applied Biosystems, Foster City, CA).

The human 143B osteosarcoma and the derived 206 ρ^0 cell lines were kindly provided by Lodovica Vergani (Department of Neurosciences, University of Padova, Padova, Italy). Cells were grown in high glucose (4500 mg/l) Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 4 mM L-glutamine, 110 mg/l sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37 °C with 5% CO_2. Medium of ρ^0 cells was supplemented with 50 mg/l uridine, essential amino acids and vitamins.

2.2. Mitochondrial membrane potential and cell death

Mitochondrial membrane potential ($\Delta\psi_m$) was measured either by epifluorescence microscopy or by flow cytometry (see below). For epifluorescence microscopy cells were seeded onto 24 mm-diameter round glass coverslips and grown for 2 days in DMEM. $\Delta\psi_m$ was measured based on the accumulation of TMRM in the presence of CsH, which inhibits the multidrug resistance pump but not the PTP [50,51]. Cells were incubated in bicarbonate- and phenol red-free DMEM supplemented with 10 mM Hepes and 1.6 μM CsH and loaded with 20 nM TMRM for 30 min at 37 °C. At the end of each experiment, mitochondria were fully depolarized by the addition of 4 μM of the protonophore FCCP. Cellular fluorescence images were acquired with an Olympus IX71/IX51 inverted microscope equipped with a xenon light source for epifluorescence illumination and with a 12-bit digital cooled CCD camera (Micromax, Princeton Instruments, Trenton, NJ). For detection of fluorescence 568 ± 25 nm bandpass excitation and 585 nm longpass emission filter settings were used. Images were collected every 3 min with an exposure time of 100 ms (6% illumination intensity) using a 40×, 1.3 NA oil immersion objective (Olympus). Data were acquired and analyzed using Cell R software (Olympus). Clusters of several mitochondria were identified as regions of interest, and fields not containing cells were taken as the background. Sequential digital images were acquired every minute, and the average fluorescence intensity of all relevant regions was recorded and stored for subsequent analysis.

For FACS analysis cells were resuspended in 135 mM NaCl, 10 mM Hepes, 5 mM $CaCl_2$ and incubated for 15 min at 37 °C with TMRM (20 nM) and CsH (1.6 μM) or with FITC-conjugated Annexin-V, to detect mitochondrial membrane depolarization (reduced TMRM staining) or phosphatidylserine exposure on the cell surface (increased FITC-conjugated Annexin-V staining) respectively. Changes in forward and side light scatter were assessed at the same time to measure alterations in cell dimension and granularity, respectively. Samples were analyzed on a FACSCanto II flow cytometer (Becton Dickinson, San Diego, CA, USA). Data acquisition and analysis were performed using FACSDiva software. Regions were designed on diagrams to evaluate the percentage of cell subpopulations as indicated in Fig. 3.

2.3. Cell lysis, fractionation, western immunoblot analysis

Total cell extracts were prepared at 4 °C in 150 mM NaCl, 20 mM Tris–HCl pH 7.4, 5 mM EDTA, 10% glycerol, 1% Triton X-100 in the presence of phosphatase and protease inhibitors (Sigma). To prepare mitochondrial extracts, cells were placed in isolation buffer (250 mM sucrose, 10 mM Tris–HCl, 0.1 mM EGTA–Tris, pH 7.4) and homogenized at 4 °C. Mitochondria were then isolated by differential centrifugation in mitochondrial isolation buffer. Unless otherwise stated, mitochondria were treated with trypsin at 4 °C for 1 h at a mitochondria:trypsin ratio of 35:1 (w/w). After trypsin inactivation with a protease inhibitor cocktail (Sigma), mitochondria were spun at 18,000 × *g* at 4 °C for 10 min. For immunoprecipitation, 4 mg of extracted proteins per reaction was incubated with antibodies conjugated to protein A-Sepharose beads (Pharmacia, Pfizer, Cambridge, MA) at 4 °C overnight. Negative controls were performed by incubating lysates on beads in the absence of primary antibodies. Samples were separated under reducing conditions on SDS-polyacrylamide gels and transferred onto Hybond-C Extra membranes (Amersham). Primary antibodies were incubated 16 h at 4 °C, and horseradish peroxidase-conjugated secondary antibodies were added for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence (Millipore). Densitometric analysis was performed with Quantity One software (Bio-Rad Laboratories).