Free Radical Biology and Medicine 67 (2014) 314–329

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original Contributions

Hypoxic preconditioning-induced mitochondrial protection is not disrupted in a cell model of mtDNA T8993G mutation-induced F1F0-ATP synthase defect: the role of mitochondrial permeability transition

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ARTICLE INFO

Article history:
Received 13 July 2013
Received in revised form
24 October 2013
Accepted 21 November 2013
Available online 27 November 2013

Keywords:
Hypoxic preconditioning
NARP
Cyclophilin-D
F1F0-ATP synthase
Mitochondria
Free radicals

ABSTRACT

Transient opening of the mitochondrial permeability transition pore plays a crucial role in hypoxic preconditioning-induced protection. Recently, the cyclophilin-D component of the mitochondrial permeability transition pore has been shown to interact with and regulate the F1F0-ATP synthase. However, the precise role of the F1F0-ATP synthase and the interaction between cyclophilin-D and F1F0-ATP synthase in the mitochondrial permeability transition pore and hypoxic preconditioning remain uncertain. Here we found that a 1-h hypoxic preconditioning delayed apoptosis and improved cell survival after stimulation with various apoptotic inducers including H_2O_2, ionomycin, and arachidonic acid in mitochondrial DNA T8993G mutation (NARP) osteosarcoma 143B cybrids, an F1F0-ATP synthase defect cell model. This hypoxic preconditioning protected NARP cybrid cells against focal laser irradiation-induced oxidative stress by suppressing reactive oxygen species formation and preventing the depletion of cardiolipin. Furthermore, the protective functions of transient opening of the mitochondrial permeability transition pore in both NARP cybrids and wild-type 143B cells can be augmented by hypoxic preconditioning. Disruption of the interaction between cyclophilin-D and F1F0-ATP synthase by cyclosporin A attenuated the mitochondrial protection induced by hypoxic preconditioning in both NARP cybrids and wild-type 143B cells. Our results demonstrate that the interaction between cyclophilin-D and F1F0-ATP synthase is important in the hypoxic preconditioning-induced cell protection. This finding improves our understanding of the mechanism of mitochondrial permeability transition pore opening in cells in response to hypoxic preconditioning, and will be helpful in further developing new pharmacological agents targeting hypoxia–reoxygenation injury and mitochondria-mediated cell death

The restoration of circulation in the tissue after prolonged ischemia results in inflammation and oxidative damage through the induction of oxidative stress [1,2] and leads to the tissue damage known as ischemic–reperfusion (IR)[1] injury. IR injuries are frequently seen in the patients with myocardial or cerebral infarction [3,4]. Transient episodes of nonlethal hypoxia before prolonged ischemia, also called hypoxic preconditioning (HPC), demonstrate a protective potential against IR injury [5–10]. At present, the exact molecular mechanisms involved in HPC-induced protection remain unclear. Studies have suggested that mitochondria play a critical role in the HPC-induced protective mechanism, which may be mediated through the modulation of the mitochondrial permeability transition pore (mPTP), a nonselective pore located on the inner mitochondrial membrane [11–13].

Abbreviations: IR, ischemia–reperfusion; HPC, hypoxic preconditioning; mPTP, mitochondrial permeability transition pore; mCa^{2+}, mitochondrial calcium; $\Delta\Psi m$, mitochondrial membrane potential; ROS, reactive oxygen species; cypD, cyclophilin-D; ATPase, F1F0-ATP synthase; mtDNA, mitochondrial DNA; NARP, mitochondrial DNA T8993G mutation; CsA, cyclosporin A; ρ°, mtDNA-less; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; AA, arachidonic acid; ROI, region of interest; BKA, bongkrekic acid; ANT, adenine nucleotide translocase; PS, phosphatidylserine; PI, propidium iodide; TMRM, tetramethylrhodamine methyl ester; DCFH-DA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; NAO, nonyl acridine orange; mPT, mitochondrial permeability transition; DCF, DCFH-DA oxidized to its fluorescent form; mROS, mitochondrial reactive oxygen species

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http://dx.doi.org/10.1016/j.freeradbiomed.2013.11.019

Two modes of mPTP opening have been described. The irreversible permanent mPTP opening allows ions and solutes to enter the mitochondrial matrix in response to various apoptotic insults (e.g., oxidative stress, mitochondrial Ca^{2+} (mCa^{2+}) overload, etc.), leading to rapid dissipation of mitochondrial membrane potential ($\Delta\Psi m$) and cell death [14–19]. In contrast, the protective transient mPTP opening modulates the release of mCa^{2+} and reactive oxygen species (ROS) when healthy cells are exposed to sublethal stress, such as HPC [20–22]. The precise regulatory mechanism of this HPC-mediated transient mPTP opening remains elusive. Recent studies suggest that the cyclophilin-D (cypD) component of the mPTP is important to HPC-induced protection [12]. Giorgio et al. demonstrated that the cypD component of the mPTP forms a complex and interacts with F1F0-ATP synthase (ATPase) [23,24], which is also an important function regulator of mitochondria during hypoxic responses [25–28]. It is still uncertain whether HPC-induced cell protection is regulated by ATPase. The role of the interaction between cypD and ATPase in HPC-induced cell protection has not been extensively investigated.

To address the above questions, we used mitochondrial DNA (mtDNA) T8993G mutation (NARP) osteosarcoma 143B cybrids, which harbor 98% mtDNA T8993G genes, as an in vitro model for cells with an ATPase defect. The mtDNA T8993G mutant (Leu156Arg) alters the assembly and stability of ATPase, compromising its enzyme activity [29,30]. We found that HPC-induced protection is well preserved in NARP cybrid cells. The protective functions of the transient opening of the mPTP in both NARP cybrids and wild-type 143B cells are augmented by HPC. The disruption of the interaction between cypD and ATPase by cyclosporin A (CsA) [24] attenuates the HPC-induced transient mPTP-mediated protection in both NARP cybrids and wild-type 143B cells. These results indicate the important role of the interaction between cypD and ATPase in HPC-induced cell protection.

Materials and methods

Establishment of NARP cybrids

The NARP cybrids were established as described previously [31]. Briefly, skin fibroblasts obtained from a patient with Leigh disease carrying the mtDNA T8993G mutation were enucleated and cytoplasmically fused with mtDNA-less (ρ°) human osteosarcoma 143B cells. The NARP and ρ° cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) supplemented with high glucose (4.5 g/ml), pyruvate (0.11 mg/ml), and uridine (0.1 mg/ml). NARP cybrids with a high mutant mtDNA-to-wild-type mtDNA ratio of approximately 98% were used for the experiments, and comparisons were made to the parental 143B cell line. Both the NARP cybrids and the 143B cells described above were kindly provided by Dr. M. Tanaka from Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan.

HPC treatment of NARP cybrids and 143B cells

All cells were grown in DMEM containing 10% (v/v) FBS supplemented with high glucose (4.5 g/ml), pyruvate (0.11 mg/ml), and uridine (0.1 mg/ml). The cells were plated onto poly-L-lysine-coated glass coverslips (Model No. 1; VWR Scientific, San Francisco, CA, USA). Experiments were performed after cells had grown to 80–90% confluence after 48–72 h in culture. To induce hypoxia, cell cultures were put in a modular incubator chamber flushed with 95% N_2 and 5% CO_2, according to manufacturer's instructions (Billups-Rothenberg, Del Mar, CA, USA). The deoxygenation reagent (CO_2 5% and O_2 less than 1%; Mitsubishi Gas Chemical, Tokyo, Japan) was placed inside the chamber. The chamber was sealed and placed into a 37 °C incubator for 1 h (HPC). After hypoxia incubation, the cells were washed with normoxic culture medium twice and were then transferred to their respective normal culture medium and returned to the 37 °C incubator with 5% CO_2 to rest. Apoptotic insult, which included H_2O_2 (5 mM)-induced oxidative stress, ionomycin (1 μM)-induced Ca^{2+} stress, arachidonic acid (AA; 100 μM)-induced lipid stress, or focal laser irradiation (100% transmission of a 561-nm laser applied 1000 times within the region of interest (ROI), $2 \times 2\ \mu m^2$), was performed after the cells had rested for 1 h. CsA (10 μM; Sigma, St. Louis, MO, USA), to break the interaction between cypD and ATPase, or bongkrekic acid (BKA; 10 μM; Sigma), to inhibit ANT (adenine nucleotide translocase, another component of mPTP), was added to all buffers and media after HPC in some experiments.

Apoptotic cell analysis

The flip-flop of phosphatidylserine (PS) from the inner to the outer plasma membrane is a common phenomenon in apoptosis. PS exposure induced by apoptotic stress in cells was detected by fluorescein isothiocyanate (FITC)-conjugated annexin V staining [32]; this was imaged as green fluorescence on the plasma membrane during the occurrence of PS externalization. Using fluorescence microscopy, the precise time point for PS exposure and cell death was carefully detected by imaging of cells dual labeled with annexin V–FITC and propidium iodide (PI) after exposure to apoptotic insult. The percentage cell death was assessed using PI/Hoechst 33342 double staining. The ratio of PI-positive/Hoechst 33342-positive (%) cells was recorded as the cell death ratio. The percentage cell death was assessed as PI-positive staining in HPC groups; the group without HPC was compared at the same time.

Cell preparation for imaging

For imaging detection, cells were grown in DMEM containing 10% FBS supplemented with high glucose (4.5 g/ml), pyruvate (0.11 mg/ml), and uridine (0.1 mg/ml). All cells were plated onto No. 1 glass coverslips for fluorescence measurements.

Chemical and fluorescent dye loading for fluorescence measurement of mitochondrial events

All chemicals were obtained from Sigma, and fluorescent dyes were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Loading conditions for each specific fluorescent probe were as follows: $\Delta\Psi m$ was detected using 300 nM tetramethylrhodamine methyl ester (TMRM); mCa^{2+} was detected using 2 μM Rhod-2/AM (Rhod-2); ROS were detected using 2 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA); cardiolipin was detected using 80 nM nonyl acridine orange (NAO); mitochondrial permeability transition (mPT) was detected using 1 μM calcein/AM (calcein) with the addition of 1 mM cobalt chloride ($CoCl_2$) to quench cytosolic calcein. All fluorescent probes were loaded at room temperature for 30 min except TMRM, which was loaded for 10 min to avoid its quenching effect. After loading, the cells were rinsed three times with Hepes-buffered saline solution (containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, and 10 mM Hepes, pH 7.4). Cells loaded with the ester form of dyes, including DCFH-DA and Rhod-2, required an additional 30–40 min of incubation after the dye loading to allow intracellular deacetylation of the dye. DCFH-DA was oxidized to its fluorescent form (DCF) upon oxidant formation after being taken up by cells and deacetylated by cellular