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Original article

Synergistic protective effect of cyclosporin A and rotenone against hypoxia–reoxygenation in cardiomyocytes

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ABSTRACT

Reperfusion of the heart after an ischemic event leads to the opening of a nonspecific pore in the inner mitochondrial membrane, the mitochondrial permeability transition pore (mPTP). Inhibition of mPTP opening is an effective strategy to prevent cardiomyocyte death. The matrix protein cyclophilin-D (CypD) is the best-known regulator of mPTP opening. In this study we confirmed that preconditioning and postconditioning with CypD inhibitor cyclosporin-A (CsA) reduced cell death after hypoxia–reoxygenation (H/R) in wild-type (WT) cardiomyocytes and HL-1 mouse cardiac cell line as measured by nuclear staining with propidium iodide. The complex I inhibitor rotenone (Rot), alone, had no effect on HL-1 and WT cardiomyocyte death after H/R, but enhanced the native protection of CypD-knocked-out (CypD KO) cardiomyocytes. Reduction of cell death was associated with a delay of mPTP opening challenged by H/R and observed by the calcein loading $CoCl_2$-quenching technique. Simultaneous inhibition of complex I and CypD increased in a synergistic manner the calcium retention capacity in permeabilized cardiomyocytes and cardiac mitochondria. These results demonstrated that protection by complex I inhibition was CypD dependent.

1. Introduction

Mitochondria are recognized as critical mediators of cardiomyocyte death when the myocardium is subjected to ischemia–reperfusion injury. During reperfusion, mitochondrial dysfunction leads to the formation of a non-selective mega-channel, the mitochondrial permeability transition pore (mPTP). The mPTP opening is triggered by calcium overload and overproduction of reactive oxygen species (ROS) [1]. This leads to the collapse of mitochondrial membrane potential, ATP deprivation and release of pro-apoptotic molecules into the cytosol which to a certain point lead to cell death [2].

Preventing mPTP opening is an efficient strategy to protect the heart against lethal ischemia–reperfusion injury [3,4]. For example, inhibition of mPTP opening via ischaemic preconditioning (PreC) and post-conditioning (PostC) decreased the myocardial infarct size after ischemia–reperfusion [3,5]. Although the molecular composition of the mPTP remains unclear, the matrix protein cyclophilin-D (CypD) is the best defined regulatory component of mPTP [6–8]. CypD genetic ablation or pharmacological inhibition with cyclosporin A (CsA) reduces the infarct size in laboratory animals submitted to myocardial ischemia–reperfusion [8,9]. Other mPTP modulators such as benzodiazepine receptor [10], hexokinase I and II [11], phosphate carrier [12], and glycogen synthase kinase-3β inhibitors [13] can regulate the mPTP opening.

Recently, we demonstrated that rotenone (Rot), a mitochondrial respiratory chain complex I inhibitor, is a more potent mPTP inhibitor than CsA in various cell types [14]. Interestingly, we also showed that tissues in which Rot alone does not appear to affect mPTP opening, such as cardiac mice mitochondria, are characterized by high expression levels of CypD, suggesting that CypD and complex I might interact to modulated mPTP opening in some conditions.

Immortalized HL-1 mouse cardiomyocyte cell line exhibits characteristics of differentiated cardiac cells but shows metabolic differences from adult cardiomyocytes [15]. In these cells, glycolytic enzyme activity is increased whereas oxidative phosphorylation is very low, especially at the level of complex I [16]. Glycolysis must serve as the main energy-providing system in HL-1. Unfortunately, the level of CypD is unknown in these cells. Our studies were also

Abbreviations: mPTP, mitochondrial permeability transition pore; PreC, preconditioning; PostC, postconditioning; CypD, cyclophilin-D; CsA, cyclosporin-A; Rot, rotenone; WT, wild-type; KO, knocked-out; H/R, hypoxia/reoxygenation; t_{mPTP50}, time to 50% mitochondrial permeability transition pore opening; CRC, calcium retention capacity.

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performed on adult wild-type (WT) and CypD-knocked-out (CypD KO) mice cardiomyocytes. Thereby, we were able to compare three cardiac models with different levels of CypD and complex I.

In the present study, we investigated whether CypD inhibition (CypD genetic ablation and CsA treatment) and/or complex I inhibition (by Rot) reduced cell death by modulating mPTP opening. These experiments were performed using an *in vitro* hypoxia–reoxygenation (H/R) adult mice cardiomyocyte model or HL-1 cell-line model with PreC and pharmacological treatment of reperfusion injury (PostC) protocols, as well as mitochondria isolated from these cells.

In this study, we present evidence that inhibition of complex I prevents cardiomyocyte death after H/R in a CypD dependent manner. Combined inhibition of complex I and CypD might represent a new therapeutic cardioprotective strategy.

2. Materials and methods

The present study was performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996). All the experimental procedures were approved by the ethic committee of Lyon I Claude Bernard University (BH-2007-07).

2.1. Animals

WT and CypD KO male mice (8–12 weeks, 20–30 g) were used throughout the study. B6129BF1/J WT mice were used as controls (Charles River, L'Arbresle, France). CypD KO mice were a generous gift from the laboratory of Stanely Korsmeyer [17].

2.2. Isolating adult murine ventricular cardiomyocytes

Adult WT and CypD KO mice were pre-medicated with heparin (100 USP units/mouse) (Sanofi Winthrop, Gentilly, France) administered intraperitoneally. Anesthesia was induced with sodium pentobarbital (70 mg/kg) (Sanofi Santé Animale, Libourne, France). A thoracotomy was performed and the heart collected. Ventricular cardiomyocytes were isolated using enzymatic digestion according to previously described procedures [13].

2.3. HL-1 cell line

Immortalized HL-1 cardiomyocyte cell line derived from mouse atrial tumors was a gift from Dr. W.C. Claycomb (Louisiana State University Medical Center). Cells were cultured in Claycomb medium (Sigma-Aldrich, Saint Louis, MO) supplemented with 10% fetal calf serum, 4 mM L-glutamine, penicillin and streptomycin (100 U–0.1 mg/ml), 0.3 mM ascorbic acid and 10 μM norepinephrine, at 37 °C in a humid atmosphere of 5% CO_2/95% air as previously described [18]. Cells were split at a mean density of 15,000/cm^2 in 35 mm Petri dishes coated with a mixture of 0.02% gelatin and fibronectin (12.5 μg/ml) and were allowed to reach 75% confluence before the experiments involving H/R.

2.4. Western Blot analysis

Cells were lysed with a lysis buffer containing in mM: Nonidet P-40 (1%), 20 Tris HCl, 138 NaCl, 2.7 $MgCl_2$, glycerol (5%), 5 EDTA, 1 Na_3O_4V, 20 NaF, 1 DTT, protease inhibitor (Sigma Aldrich) and phosphatase inhibitors (Roche diagnostic, Mannheim, Germany) according to the manufacturer's instructions. Protein concentration was normalized by determination of the total protein concentration by BCA assay (Bicinchoninic acid, Thermo scientific, Rockford, IL) method. Per lane, 15 μg of total protein migrate in sodium dodecyl sulfate, 12% polyacrylamide gel (SDS-PAGE). After migration, proteins were blotted to PVDF membrane by electrotransfer. Proteins were detected after labeling by specific primary antibodies revealed by secondary HRP coupled antibodies (HRP-anti-rabbit and HRP-anti-mouse from GE Healthcare, Orsay, France). The primary antibodies used were: mouse anti-VDAC (1:1000) and mouse anti-CypD (1:2000) AP1035 from Calbiochem (San Diego, CA). The revelation was obtained by addition of reaction substrate (ECL plus Kit western blotting detection systems from GE Healthcare, Pittsburgh, PA). Evaluation of protein amount was determined by using ImageLab software from BioRad (Hercules, CA). Integrated density of each band was converted to arbitrary unit to evaluate the amount of revealed protein.

2.4.1. Oxidative phosphorylation

Mitochondrial oxygen consumption was measured at 25 °C using a Clark-type oxygen electrode. Permeabilized cardiomyocytes or HL-1 (150 μg protein) with digitonin (40 μM) were incubated in 2 ml of respiration buffer containing, in mM: 100 KCl, 50 MOPS, 1 EGTA, 5 KH_2PO_4, and 1 mg/ml defatted BSA. Glutamate/malate/pyruvate (5 mM each) were used as electron donors to complex I in the electron transport chain. Respiration state 3 is initiated with addition of 1 mM ADP and expressed as nmol O_2 min^{-1} mg protein^{-1}.

2.5. Time-lapse microscopy

For hypoxia–reoxygenation and cell death quantification, cells were placed in a thermostated chamber (37 °C) mounted on the stage of a IX50 Olympus microscope (Olympus, Tokyo, Japan), as previously described [13]. The chamber was connected to a constant stream of O_2 (21%), N_2 (74%) and CO_2 (5%). Oxygen in the solution was measured using a fiber optic sensor system (Ocean Optics, Inc., Dunedin, FL). A 12-bit cooled CCD camera (Orca-R^2, Hamamatsu, Japan) allowed image acquisition. Image acquisition and analysis was performed with ImageJ software (NIH, Bethesda, MD).

2.6. Hypoxia–reoxygenation protocol

The cells were perfused with a Tyrode's solution containing in mM: 130 NaCl, 5 KCl, 10 HEPES, 1 $MgCl_2$, 1 $CaCl_2$ and 10 glucose, pH 7.4, at 37 °C for a 10 min-stabilization period. In order to induce hypoxia, the bubbling gas was replaced by O_2 free bubbled Tyrode and under a constant stream of the same gas, composed of 95% N_2

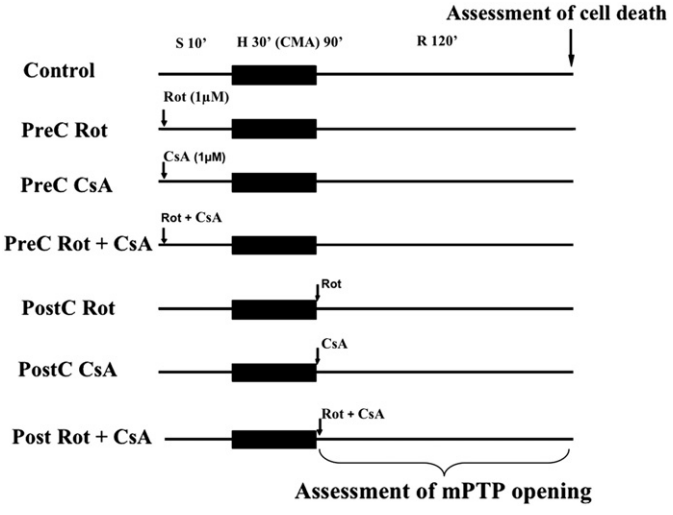

Fig. 1. *Experimental protocol (hypoxia–reoxygenation).* After a 10-min stabilization (S) period all groups underwent 30 (cardiomyocytes) or 90 min (HL-1) of hypoxia followed by 120 min of reoxygenation. H: hypoxia; R: reoxygenation; Rot: rotenone; CsA: cyclosporin A; PreC: preconditioning by injection of Rot (1 μM) or CsA (1 μM) or Rot + CsA 10 min before hypoxia; PostC: postconditioning or pharmacological treatment of reperfusion injury by injection of Rot (1 μM) or CsA (1 μM) or Rot + CsA at the beginning of reoxygenation. Cell death was quantified at the end of hypoxia–reoxygenation and mPTP opening was followed during reoxygenation period.