Biochemistry and Biophysics Reports 7 (2016) 33–38

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

Partial contribution of mitochondrial permeability transition to *t*-butyl hydroperoxide-induced cell death

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A R T I C L E I N F O

Article history:
Received 9 September 2015
Received in revised form
2 May 2016
Accepted 6 May 2016
Available online 9 May 2016

Keywords:
Cell death
Mitochondria
Mitochondrial permeability transition pore
Oxidative stress
t-butyl hydroperoxide

A B S T R A C T

Mitochondrial permeability transition (MPT) is thought to determine cell death under oxidative stress. However, MPT inhibitors only partially suppress oxidative stress-induced cell death. Here, we demonstrate that cells in which MPT is inhibited undergo cell death under oxidative stress. When C6 cells were exposed to 250 μM *t*-butyl hydroperoxide (*t*-BuOOH), the loss of a membrane potential-sensitive dye (tetramethylrhodamine ethyl ester, TMRE) from mitochondria was observed, indicating mitochondrial depolarization leading to cell death. The fluorescence of calcein entrapped in mitochondria prior to addition of *t*-BuOOH was significantly decreased to 70% after mitochondrial depolarization. Cyclosporin A suppressed the decrease in mitochondrial calcein fluorescence, but not mitochondrial depolarization. These results show that *t*-BuOOH induced cell death even when it did not induce MPT. Prior to MPT, lactate production and respiration were hampered. Taken together, these data indicate that the decreased turnover rate of glycolysis and mitochondrial respiration may be as vital as MPT for cell death induced under moderate oxidative stress.

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1. Introduction

Mitochondria utilize the pH gradients and membrane potentials that exist across their inner membranes to transport charged molecules and ions. To maintain the pH gradients and membrane potentials, it is necessary for the permeability of the inner mitochondrial membrane to be kept low. However, when mitochondria undergo Ca^{2+} overloading or oxidative stress, the inner membranes become permeable to solutes with molecular masses that are below approximately 1.5 kDa; this is known as mitochondrial permeability transition, MPT) [1]. Therefore, upon MPT, mitochondrial function changes drastically, and the intracellular environment is altered. As such, MPT is thought to be involved in cellular damage [2,3] or intracellular signal transduction [4,5]. MPT can be blocked when the mitochondrial peptidylprolyl cis-trans isomerase (PPIase) cyclophilin D is inhibited [6] or its gene is ablated [7,8], thus indicating that MPT is a protein-dependent process.

Cellular dysfunction due to reactive oxygen species (ROS) has been observed in diseases including ischemia reperfusion injury and neurodegenerative disorders [9]. ROS suppress energy production by hampering glycolysis [10,11] and mitochondrial function [12–16]. In particular, intensive studies on ROS-induced MPT have demonstrated that MPT inhibition by cyclosporin A (CsA) increases cells viability under oxidative stress. However, CsA only partially suppresses ROS-induced cell death [17,18]. In these cases, it is unclear whether cell death is accompanied by MPT or not.

To detect MPT in cells, we can monitor the translocation of small molecules across the inner membrane by using hydrophilic fluorescent dye calcein [13,14] or radiolabeled 2-deoxyglucose [15,16]; these are smaller than the MPT cutoff size of 1.5 kDa. Although calcein is suitable for simultaneous measurements of mitochondrial membrane potential and the occurrence of MPT, we must consider the effects of ROS on the mitochondrial uptake of calcein AM and the quenching of calcein fluorescence in mitochondria. In this study, we successfully excluded these effects of ROS and demonstrated that mitochondria became depolarized by

Abbreviations: AM, acetoxymethyl ester; CsA, cyclosporin A; DMEM, Dulbecco's modified Eagle's medium; ΔΨm, mitochondrial membrane potential; FBS, fetal bovine serum; HBS, HEPES-buffered saline; MPT, mitochondrial permeability transition; *t*-BuOOH, *t*-butyl hydroperoxide; TMRE, tetramethylrhodamine ethyl ester; PPIase, peptidylprolyl cis-trans isomerase; ROS, reactive oxygen species

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http://dx.doi.org/10.1016/j.bbrep.2016.05.005
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ROS even when MPT was inhibited by CsA. This result implies that ROS significantly decreases ATP production even when MPT does not occur and further promotes necrotic cell death.

2. Experimental procedures

2.1. Reagents

Calcein AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Tetramethylrhodamine ethyl ester (TMRE) was purchased from Invitrogen Corporation (Carlsbad, CA). CsA and digitonin were obtained from Sigma-Aldrich Co. (St. Louis, MO). All other high purity chemicals were commercially available.

2.2. Cell Cultures

C6 glioma cell lines overexpressing wild-type or PPIase-deficient mutants (R97A) of cyclophilin D and control cells transfected with the corresponding empty vector were obtained, as described previously [19]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 µg/mL of geneticin in a humidified incubator at 37 °C and 5% CO_2. After enzymatic dissociation with trypsin, cells were plated in culture dishes (diameter, 35 mm) at a concentration of 2.8×10^4 cells/cm^2. The cells were cultured under the above conditions for 2 days prior to measurements.

2.3. Fluorescence staining and the addition of t-BuOOH

In order to observe the changes in the plasma membrane integrity and $\Delta\Psi$m by exposing cells to *t*-BuOOH, C6 glioma cells were loaded with 1 µM calcein AM [20] and 100 nM TMRE [21,22], respectively, for 30 min in DMEM without serum at 37 °C. Cells were then washed with DMEM and incubated with 250 µM *t*-BuOOH [13,23] in DMEM with 100 nM TMRE and 10% FBS. Immediately before microscopic observation, the medium was replaced with HEPES-buffered saline (HBS) (10 mM HEPES, 120 mM NaCl, 4 mM KCl, 0.5 mM $MgSO_4$, 1 mM NaH_2PO_4, 4 mM $NaHCO_3$, 25 mM glucose, 0.1% bovine serum albumin, pH 7.4) with 1.2 mM $CaCl_2$ and 100 nM TMRE.

In order to observe MPT in *t*-BuOOH treated cells, the calcein-digitonin technique [24] was used with slight modification. First, to trap calcein in the mitochondria, C6 glioma cells in culture dishes were incubated with 1 µM calcein AM and 100 nM TMRE for 30 min at 37 °C in DMEM with 0.005% cremophor. The calcein AM and cremophor were removed by washing cells with DMEM. The cells were then incubated with 250 µM *t*-BuOOH in DMEM with 100 nM TMRE and 10% FBS. In order to examine the effects of CsA, CsA was added to a final concentration of 5 µM after washing cells and before the addition of *t*-BuOOH, and this remained in the media throughout the remaining procedures. After the appropriate incubation time, the cells were detached from the culture dishes with 0.05% trypsin in phosphate-buffered saline (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.44 mM ethylenediaminetetraacetic acid, pH 7.4) with 250 µM *t*-BuOOH. After removing trypsin by centrifugation at $126 \times g$ for 10 min, the cell pellet was suspended in HBS at a density of 3×10^6 cells/mL. The suspension was divided into 3 aliquots. The first was maintained in HBS with 250 µM *t*-BuOOH (HBS(tB)). The second was incubated with 20 µM digitonin in HBS(tB) to remove the cytosolic calcein by permeabilizing the plasma membranes. The third was incubated with 100 nM TMRE in HBS(tB) to observe $\Delta\Psi$m. Immediately before microscopic observations, the cells were transferred to a hemocytometer with a 0.17-mm thick coverslip. Calcein fluorescence in the cells of the second aliquot was observed for 15–25 min after the addition of digitonin, as mitochondrial calcein fluorescence was stable during this period. After measuring the second aliquot, we measured the first and third aliquots to confirm plasma membrane integrity and the maintenance or loss of the $\Delta\Psi$m. *t*-BuOOH was added to control cells 15 min before their detachment from the dishes to compensate for the possible bleaching of calcein fluorescence. After detachment, all procedures were performed at 25 °C and completed within 30 min.

2.4. Imaging and Analysis of Calcein and TMRE Fluorescence

In order to obtain the fluorescence images, we used an inverted epifluorescence microscope (IX-70; Olympus Corporation, Tokyo, Japan). The magnification of the objective lens was 20 times (Uapo20X/340, NA=0.75; Olympus Corporation). Calcein fluorescence was monitored using a 75-W xenon lamp through a 20-nm bandpass filter centered at 480 nm. The illumination intensity was reduced to 6% with a neutral density filter. Light emitted between 515 and 550 nm was collected with a cooled CCD camera (Sensicam QE, PCO AG; Kelheim, Germany). For the TMRE fluorescence, excitation was achieved with a 15-nm bandpass filter centered at 535 nm. Fluorescence > 580 nm was collected [25,26]. The illumination intensity was reduced to 25% with a neutral density filter. All images were obtained with 2×2 binning pixels and an exposure time of 1 s. The fluorescence readouts were digitized to 12 bits and analyzed with image-processing software (MetaMorph; Molecular Devices, Inc., Sunnyvale, CA). In order to analyze the fluorescence intensity of intracellular calcein, we identified the outline of each cell in the transmitted image and obtained the average intensity of the fluorescence within the region surrounded by the outline. The background intensity of the fluorescence was obtained as the average intensity within a region where the intensity of the fluorescence was not affected by the cells stained with calcein. This was subtracted from the intensity of the calcein fluorescence in each cell.

2.5. Measurements of cell respiration

Prior to the measurements of cell respiration, cells grown in culture dishes were incubated with 250 µM *t*-BuOOH in DMEM with 10% FBS for 15 min at 37 °C. The cells were then detached from the culture dishes and collected, as described in the section on the calcein-digitonin technique. Cells not incubated with *t*-BuOOH were used as a control. During cell respiration measurements, the cells were suspended in HBS(tB) with 1.2 mM $CaCl_2$ at a concentration of 1.5×10^7 cells/mL. To evaluate cell respiration, oxygen concentrations were measured with a Clark-type oxygen electrode (Iijima-Denshi, Aichi, Japan) for 10 min at 37 °C. These measurements were completed within 40 min after the addition of *t*-BuOOH to cells grown in culture dishes.

2.6. Measurements of ATP and lactate production

Prior to determination of lactate levels, cells were incubated in DMEM without FBS for 20 min. When rotenone and/or *t*-BuOOH were added, these reagents were present during this incubation. Cells were then washed twice with DMEM without FBS and were incubated in the medium for 30 min in the presence or absence of 600 nM rotenone and/or 250 µM *t*-BuOOH. After this incubation, lactate in the medium was determined enzymatically by a test kit (L-lactic acid, Boehringer Mannheim, Germany). The amount of cells in a dish was determined as protein amount using a protein assay with BSA as a standard.

For determination of ATP, cells were plated in 96-well microplates at a concentration of 2500 cells/well and cultured in DMEM with 10% FBS for 48 h at 37 °C in a CO_2 incubator. When *t*-BuOOH

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