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Mitochondrial m-calpain opens the mitochondrial permeability transition pore in ischemia–reperfusion

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ABSTRACT

Background/objectives: : Opening of the mitochondrial permeability transition pore (mPTP) is involved in ischemia–reperfusion injury. Isoforms of Ca^{2+}-activated cysteine proteases, calpains, are implicated in the development of myocardial infarction in ischemia–reperfusion. Growing evidence has revealed the presence of calpains in the mitochondria. We aimed to characterize mitochondrial calpains in the rat heart and to investigate the roles of calpains in mPTP opening after ischemia–reperfusion.

Methods and results: : Western blotting analysis showed the expression of μ-calpain, m-calpain and calpain 10 in mitochondria isolated from male Sprague-Dawley rats, but casein zymography detected only m-calpain activity. Subcellular fractionation of mitochondria demonstrated the distribution of m-calpain to the matrix fraction. Addition of >500 μM of Ca^{2+} to isolated mitochondria induced mitochondrial swelling, reflecting mPTP opening, and calpain activation. Ca^{2+}-induced mitochondrial swelling was inhibited partially by the calpain inhibitor calpeptin. These results support a partial contribution of calpain in the opening of the mPTP. The addition of Ca^{2+} to the mitochondria induced inactivation of complex I of the electron transport chain, and cleavage of the ND6 complex I subunit, which were inhibited by calpeptin. Mitochondria isolated from rat hearts that underwent 30 min of coronary occlusion followed by 30 min of reperfusion showed activation of mitochondrial calpains, ND6 cleavage, complex I inactivation, and mPTP opening, which were inhibited by pretreatment with calpain inhibitor 1.

Conclusions: : We demonstrated for the first time the presence of mitochondrial matrix m-calpain, and its contribution to complex I inactivation and mPTP opening after postischemic reperfusion in the rat heart.

1. Introduction

The mitochondrial permeability transition pore (mPTP) is a non-specific pore in the inner mitochondrial membrane that is permeable to small molecules. Opening of the mPTP during ischemia–reperfusion (IR) induces apoptosis through the mitochondrial release of proapoptotic proteins and necrosis through ATP depletion (for a review, see [1]). Inhibitors of mPTP such as cyclosporine A (CsA) [2], sanglifehrin-A [3], and NIM811 [4], limit the size of myocardial infarction (MI) in an ex vivo IR injury model. mPTP opening is induced physiologically by Ca^{2+} and is enhanced by mitochondrial Ca^{2+} overload in IR. The uniporter blocker Ru360, attenuates mitochondrial Ca^{2+} uptake and prevents IR injury through inhibition of mPTP opening in vitro [5] and ex vivo [6].

Calpains, Ca^{2+}-activated cysteine proteases, are thought to contribute to the development of MI in IR [7]. Ca^{2+} overload after reperfusion induces the activation of calpains, which renders the membrane fragile through proteolysis of the cytoskeletal protein fodrin [8]. Several calpain inhibitors reduce MI size in IR injury models [9–12]. Among the 15 calpain isoforms, 10 isoforms, including conventional calpain 1 (μ-calpain) and calpain 2 (m-calpain), are expressed in the heart [13]. Although calpains are generally thought to reside in the cytosol, in a few years, growing evidence has supported a role for mitochondrial calpains in mitochondrial dysfunction. In bovine pulmonary artery smooth muscle, the Ca^{2+} ionophore A23187 induces the activation of μ-calpain in the mitochondrial intermembrane space (IMS), thereby cleaving the Na^{+}/Ca^{2+} exchanger (NCX) in the mitochondrial inner membrane (IM) [14]. In rat liver mitochondria, μ-calpain induces the truncation and subsequent cytosolic release of apoptosis-inducing factor (AIF) in the mitochondrial IMS, whereas m-calpain cleaves voltage-dependent anion channels (VDACs) in the mitochondrial outer membrane (OM) [15]. In the rat renal mitochondrial matrix,

Abbreviations: AIF, apoptosis-inducing factor; AK2, adenylate kinase 2; BSA, bovine serum albumin; CsA, cyclosporine A; DMF, N,N-dimethylformamide; IM, inner membrane; IMS, intermembrane space; IR, ischemia–reperfusion; LAD, left anterior descending coronary artery; MI, myocardial infarction; mPTP, mitochondrial permeability transition pore; NCX, Na^{+}/Ca^{2+} exchanger; OM, outer membrane; PDH, pyruvate dehydrogenase; SR, sarcoplasmic reticulum; TBS-T, Tris-buffered saline with Tween-20; VDAC, voltage-dependent anion channel.

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calpain 10 induces Ca^{2+}-induced mitochondrial swelling (mPTP opening) and inhibition of complex I activity on the electron transport chain (ETC.) through cleavage of complex I subunits (ND6 and NDUFC2) [16]. In heart mitochondria, only one report showed the presence of μ-calpain in the mitochondrial IMS [17]. However, the role of mitochondrial calpains in mPTP opening, mitochondrial dysfunction, and MI development has not been studied in the heart after IR.

Recently, we found that enhanced Ca^{2+} loading in the sarcoplasmic reticulum (SR) blocks cytosolic Ca^{2+} overload and fodrin proteolysis after IR [18]. However, the SR-loaded Ca^{2+} was transferred to the mitochondria, resulting in mitochondrial Ca^{2+} overload, acceleration of mPTP opening, and MI development [18]. These findings led us to hypothesize that mitochondrial calpains are activated through SR-mitochondria Ca^{2+} transfer, and are implicated in mPTP opening and MI development after IR. To test this hypothesis, we characterized mitochondrial calpains and investigated their targets and roles in mPTP opening in rat hearts after IR.

2. Materials and methods

This study was performed according to the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of the University of Tokyo. The animal experiment protocols are summarized in Fig. S1 (Supplementary Data).

2.1. Isolation and subfractionation of mitochondria

All of the procedures were carried out at 4 °C. Cardiac mitochondria were isolated from 8-week-old male Sprague-Dawley rats using a Mitochondria Isolation Kit for Tissue and Cultured Cells (#K288-50; BioVision, Milpitas, CA), and the isolated mitochondria underwent subfractionation according to Ozaki et al. [19]. Briefly, the isolated mitochondria were suspended in 2 volumes of 20 mM of potassium phosphate buffer containing 0.2 mg/ml of bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) at pH 7.4 and allowed to stand at 4 °C for 1 h. The suspension was centrifuged at 3000 ×*g* for 10 min, and the supernatant was centrifuged at 105,000 ×*g* for 30 min. The pellet and supernatant were defined as the OM and IMS fractions, respectively. The 3000 ×*g* pellet was resuspended in potassium phosphate buffer, sonicated (15 s, 4 times), and centrifuged at 77,000 ×*g* for 60 min. The pellet and supernatant were defined as the IM and matrix fractions, respectively.

The mitochondria and their subfractions were used immediately for assays for both mitochondrial swelling and Complex I activity. The residual samples were stored at −80 °C until use for other assays. Protein concentrations of these fractions were determined with a Coomassie Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) after solubilization with 1 M NaOH.

Human mitochondria were prepared from post-mortem heart tissue collected in two forensic autopsies performed at Kyoto Prefectural University: a 21-year-old woman at 29 h after death (case 1) and a 48-year-old woman at 40 h after death (case 2). The use of autopsy materials was approved by the Ethical Review Board of Kyoto Prefectural University. Mitochondrial isolation was performed immediately after heart tissue collection.

2.2. Percoll/OptiPrep density gradients

The isolated mitochondria were layered over a combination gradient of 6% (v/v) Percoll® (Santa Cruz Biotechnology, Santa Cruz, CA) and 17% and 35% (v/v) OptiPrep™ (Axis-Shield Poc AS, Oslo, Norway) according to Ogbi et al. [20]. After centrifugation at 50,000 ×*g* for 30 min, the gradient was fractionated into 12 tubes.

2.3. Western blot analysis

The mitochondria and their subfractions were solubilized in Laemmli sample buffer and subjected to SDS-PAGE on either 12.5% gels for the detection of voltage-dependent ion channel 1 (VDAC1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and pyruvate dehydrogenase (PDH), 7.5% gels for the detection of AIF, m-calpain, μ-calpain, and calpastatin, or 15% gels for the detection of VDAC1, calpain 10, adenylate kinase 2 (AK2), and ND6. Blots were probed with antibodies specific to AK2, calpastatin (Santa Cruz Biotechnology), VDAC1, calpain 10 (abcam, Tokyo, Japan), PDH, ND6, NDUFV2 (Sigma-Aldrich, St. Louis, MO), AIF (Merck Millipore, Billerica, MA), GAPDH (Applied Biological Materials, Richmond, BC), DAP13 (Aviva System Biology, San Diego, CA), NDUFS7 (GeneTex, Hsinchu City, Taiwan), or m-calpain and μ-calpain (kindly provided by K. Inomata, Tokyo Metropolitan Institute of Gerontology). These primary antibodies were diluted 1000-fold with 1% BSA in Tris-buffered saline with Tween-20 (BSA/TBS-T). Immune complexes were detected with horse radish peroxidase-conjugated goat antibodies to mouse or rabbit IgG (1:5000 dilution with 1% BSA/TBS-T; Promega, Madison, WI) and chemiluminescence reagents (Western Lightning-ECL; PerkinElmer, Waltham, MA). Band intensities were measured using ImageQuant™ LAS 4000 mini (GE Healthcare, Little Chalfont, UK).

2.4. Zymography

Casein zymography was performed according to the protocol described by Arthur and Mykles [21]. Briefly, FITC-casein (0.45 mg/ml; AnaSpec, Fremont, CA) was copolymerized with the separating gel containing 10% (w/v) acrylamide (acrylamide:bisacrylamide ratio = 74:1) and 225 mM of Tris–HCl (pH 8.8), and then a stacking gel containing 4.8% acrylamide and 125 mM of Tris–HCl (pH 6.8) was poured. After pre-running of the gel at 125 V for 30 min, mitochondrial lysate (10 μg) was loaded and separated at 125 V for 2 h. The gel was incubated with either 5 mM of $CaCl_2$ or 5 mM of EDTA (for control) in 50 mM Tris–HCl (pH 7.3) containing 10 mM of 2-mercaptoethanol for 30 min. The caseinolytic activity was viewed on a UV transilluminator. The proteins on the Ca^{2+}(+)-zymogram were transferred to a nitrocellulose membrane for Western blot analysis of m-calpain.

2.5. Evaluation of mPTP opening

Opening of the mPTP was evaluated by swelling of isolated cardiac mitochondria [18]. The isolated mitochondria (approximately 0.2 mg protein) were preincubated at 30 °C for 10 min in medium containing 110 mM of KCl, 20 mM of MOPS, 10 mM of Tris–HCl, 0.5 μM of rotenone, and 0.5 μM of antimycin A (pH 7.4). To determine Ca^{2+}-induced mitochondrial swelling, $CaCl_2$ was added and absorbance at 520 nm (A520) was monitored for 15 min using a microplate reader (Infinite® 200 PRO; Tecan, Männedorf, Switzerland).

2.6. Calpain activity assay

The mitochondria (0.2 mg protein) were incubated with 10 μM of t-BOC-LM-CMAC (cell-permeable fluorogenic calpain substrate; Life Technologies Corporation, Carlsbad, CA) at room temperature for 30 min, and then were centrifuged at 10,000 ×*g* for 15 min to remove the substrate. The mitochondrial pellet was resuspended, and the fluorescence of hydrolyzed CMAC was measured using a microplate reader (Infinite® 200 PRO; Tecan) with excitation at 360 nm and emission at 460 nm.

2.7. Exogenous m-calpain treatment

The mitochondria (30 μg protein) were sonicated 3 times for 30 s with an interval of 30 s on ice and incubated with either 1, 5, or 10 μg