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μ-Calpain mediated cleavage of the Na^+/Ca^{2+} exchanger in isolated mitochondria under A23187 induced Ca^{2+} stimulation

Pulak Kar, Tapati Chakraborti, Krishna Samanta, Sajal Chakraborti *

Department of Biochemistry and Biophysics, University of Kalyani, Kalyani 741 235, West Bengal, India

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ABSTRACT

Treatment of bovine pulmonary artery smooth muscle mitochondria with the calcium ionophore, A23187 (0.2 μM) stimulates μ-calpain activity and subsequently cleaves Na^+/Ca^{2+} exchanger (NCX). Pretreatment of the A23187 treated mitochondria with the calpain inhibitors, calpeptin or MDL28170 or with Ca^{2+} chelator, EGTA does not cleave NCX. Treatment of the mitochondria with A23187 increases Ca^{2+} level in the mitochondria, which subsequently dissociates μ-calpain–calpastatin association leading to the activation of μ-calpain. Immunoblot study of the A23187 treated mitochondria with the NCX polyclonal antibody indicates the degradation of mitochondrial inner membrane NCX (110 kDa) resulting in the doublet of ~54–56 kDa NCX fragments. Moreover, *in vitro* cleavage of mitochondrial purified NCX by mitochondrial purified μ-calpain supports our conclusion. This cleavage of NCX may be interpreted as the main cause of Ca^{2+} overload and could lay a key role in the activation of apoptotic process in pulmonary smooth muscle.
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Introduction

Mitochondria are known to be involved in Ca^{2+} homeostasis and signaling, as well as in cell death, aging and diseases [1]. They can sequester large amounts of Ca^{2+} and participate in regulating amplitude and shape of cytosolic Ca^{2+} transients [1]. Excessive Ca^{2+} accumulation in mitochondria can trigger cell death and apoptosis [2].

In mitochondria, Ca^{2+} is taken up via a ruthenium-red sensitive uniporter driven by the proton electrochemical gradient and Ca^{2+} efflux is mainly mediated by Na^+/Ca^{2+} exchanger (NCX), thus leading to the concept of a continuous Ca^{2+} recycling across the mitochondrial membrane causing reciprocal changes in Ca^{2+} levels in cytosol and mitochondria [3]. Significant contribution of NCX-mediated Ca^{2+} release from mitochondria has been shown in shaping $[Ca^{2+}]_i$ responses under both physiological and pathophysiological conditions [3]. The increase in $[Ca^{2+}]_i$ causes an increase in calpain activity, which subsequently degrades some intracellular proteins by limited proteolysis. This effect could be more damaging than other reversible calcium dependent processes such as phosphorylation and dephosphorylation [4]. Nicotera et al. [5] demonstrated that inhibition of Ca^{2+} efflux from cells is sufficient to cause a sustained intracellular Ca^{2+} elevation and the demise of cells by activating Ca^{2+}-dependent hydrolytic enzymes including members of the calpain protease family.

Calpains are a family of Ca^{2+} activated cysteine proteases including ubiquitous and tissue specific isoforms that cleave their substrate proteins at discrete sites to modulate activity [6]. While calpains are a 14 members' family, the best characterized and predominant calpains are the classical m- and μ-calpains, both of which are ubiquitously expressed enzymes [7]. Their physiological roles have not been fully elucidated but include cell motility, cell differentiation, membrane fusion, platelet activation, and signal transduction [8]. Also extensive investigations are the pathological roles of calpains in cell death, where calpain cleaves key structural proteins and contributes to the release of proteins that regulate cell death, for example, apoptosis inducing factor (AIF) [6]. The interaction of the endogenous inhibitor calpastatin with calpain prevents both activation and catalytic activity of calpain [8]. *In vitro* studies have shown that calpain and calpain fragments bind to calpastatin and that calpastatin fragments bind to calpain in the presence of Ca^{2+} [9]. Consequently, the level of calpastatin is considered to play a critical role in preventing calpain mediated catabolism [10]. The level of calpastatin, which is also a suicidal substrate of calpain, is decreased by calpain-mediated proteolysis [11].

Production of hydrogen peroxide (H_2O_2) during oxidative phosphorylation [12], inflammation [13], and ischemia reperfusion injury to cells and tissues [14] can cause oxidative stress, and subsequently Ca^{2+} overload in the systems. The rise in $[Ca^{2+}]_i$ in turn activates calcium dependent protease, calpain, which induces apoptosis [15]. The Ca^{2+} ionophore, A23187 transports Ca^{2+} across the cell membrane and increases $[Ca^{2+}]_i$ [15]. Bano et al. [16] demonstrated that in brain ischemia, μ-calpain cleaves plasma membrane NCX. μ-Calpain also cleaves a variety of regulatory

* Corresponding author. Fax: +91 33 25828282.
E-mail address: sajal_chakraborti@yahoo.com (S. Chakraborti).

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proteins, such as, L-type Ca^{2+} channels in hippocampal neurons [17], caspase-3 during 3-nitropropionic acid induced striatal degeneration [18] and troponin T in cardiac ischemia [19]. These observations raise a possible role of calpain on the proteolytic cleavage of NCX and subsequently Ca^{2+} overload in mitochondria.

In the present study, we report that the calcium ionophore, A23187 induces Ca^{2+} uptake in mitochondria $[Ca^{2+}]_m$ isolated from bovine pulmonary artery smooth muscle. The increase in $[Ca^{2+}]_m$ causes dissociation of associated μ-calpain and calpastatin and consequently activates the μ-calpain in the mitochondria, which cleaves the mitochondrial inner membrane 110 kDa NCX into two fragments, resulting in the doublet of 54–56 kDa bands along with the 110 kDa band. To the best of our knowledge, this is first report regarding μ-calpain mediated cleavage of the NCX during increase in Ca^{2+} level in mitochondria. We suggest that the proteolysis of NCX by μ-calpain plays an important role in an increase in $[Ca^{2+}]_m$ leading to induction of apoptotic processes in pulmonary artery smooth muscle.

Materials and methods

Materials

Percoll was purchased from Amersham Biosciences (Piscatway, NJ). A23187, PMSF, DTT, EGTA, Triton X-100, sucrose, $CaCl_2$ and MOPS were the products of Sigma Chemical Co (St. Louis, MO). CGP-37157 was from Tocris (Ellisville, MO). Ruthenium red (RR) was purchased from ICN Pharmaceuticals (Plainville, NY). Fura 2-AM and SBFI-AM was obtained from Molcular Probes (Eugene, OR). SLLVY-AMC was purchased from Bachem (King of Prussia, PA). 1,2-Diheptanoyl-sn-phosphatidylcholine (DHPC) and dioleoyl-phosphatidylcholine (DOPC) were obtained from Avanti polar lipids (Alabaster, AL). Calpeptin, mouse anti β1-integrin, mouse anti Lamp-1 were the products of Calbiochem (Sanfrancisco, CA) and MDL28170 was from Hoechst Marion Roussel (Bridgewater, NJ). Hammersten casein was purchased from U.S. Biochemical Corp. (Cleveland, OH). Rabbit polyclonal voltage-dependent anion channel (VDAC) antibody was the product of Affinity Bioreagents (Golden, CO); rabbit polyclonal anti-mannosidase II was purchased from U.S Biological (Swampscott, MA); mouse anti-calnexin was the product of BD Transduction Laboratories (San Jose, CA). Polyclonal antibody anti-lactate dehydrogenase (LDH) (host goat) and rabbit polyclonal antibody to histone H3 were, respectively, the products of Fitzerald Industries International, Inc., (Concord, MA) and Abcam Ltd., (Cambridge, UK). Rabbit polyclonal antibody of μ-calpain was kindly donated by Prof. K.K.W. Wang (University of Florida, Gainesville, FL). Rabbit polyclonal antibody of calpastatin was kindly donated by Prof. J. Takano (RIKEN Brain Science Institute, Saitama, Japan). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from Zymed (San Francisco, CA) and Jakson Immunoresearch (West Grove, PA), respectively. DEAE cellulose and phenylsepharose were the products of Amersham Pharmacia Biotech (Piscataway, NJ). DEAE-TSK (ToyoPearl) 650S was obtained from Supelco (Bellefonte, PA). All other used chemicals and reagents were the products of Sigma Chemical Co. (St. Louis, MO).

Methods

Isolation of pulmonary artery smooth muscle tissue

Bovine pulmonary artery smooth muscle tissue was collected and characterized by following the previously described procedure [20].

Isolation of mitochondria from pulmonary artery smooth muscle tissue

Mitochondria from the bovine pulmonary artery smooth muscle tissue were isolated according to our previously described protocol [20]. Briefly, the smooth muscle tissue was homogenized with a cyclomixer in ice-cold mitochondrial isolation buffer (MIB) (200 mM mannitol, 100 mM sucrose, 20 mM Hepes pH 7.2). The homogenate was centrifuged at 2000*g* for 10 min at 4 °C. The supernatant was collected and the pellet was resuspended in MIB and centrifuged again. The pooled supernatants were centrifuged at 12,500*g* for 15 min at 4 °C and the pellet was resuspended in MIB and mitochondria were isolated by discontinuous percoll density gradient centrifugation [20].

Electron microscopic study

Electron microscopic study of the mitochondria isolated from bovine pulmonary artery smooth muscle tissue homogenate was performed by following the procedure of Baudhuin [21] with some modifications [20]. Briefly, the samples were prefixed with 3% glutaraldehyde in 0.1 M Na–cacodylate buffer (pH 7.2), post fixed with 1% buffered osmium tetroxide and then centrifuged. The pellets were then dehydrated through graded series of ethanol and embedded in Agar 100 resin. Ultrathin sections were cut in a Leica ultracut UCT ultramicotome, stained with a saturated solution of uranyl acetate and 0.2% lead citrate (pH 12.0). Grids were examined in a FEI Tecnai 12 Bio Twin Transmission Electron Microscope fitted with a SIS (SIS GmbH, Germany) CCD camera.

Western blotting

Western blottings were carried out by following the procedure described by Towbin et al. [22] with some modifications. Briefly, sample buffer was added to the protein samples and all lanes were loaded with the same amount of protein (10–50 μg). Then the samples were separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were incubated for 1 h in 5% non-fat milk in 50 mM Tris–saline containing 0.05% Tween 20 at pH 7.5 (TTBS). The membranes were incubated overnight in the primary antibody in TTBS at 22 °C; then the membranes were rinsed three times in TTBS and incubated in horseradish peroxidase conjugated appropriate secondary antibodies. The membranes were then washed three times with TTBS (20 min each), and then developed with 0.2 mM 4-chloro-1-naphthol.

Co-immunoprecipitation of μ-calpain and calpastatin

Five micrograms of the μ-calpain antibody (or calpastatin antibody) were incubated with 50 μl of protein A/G agarose beads for 40 min at 4 °C as described previously [23]. The calpain antibody (or calpastatin antibody) was substituted with IgG in controls. The protein A/G agarose-anti-μ-calpain (or anti-calpastatin) complex was washed three times with PBS containing 0.1% Triton X-100; then incubated overnight at 4 °C with the Triton extracted mitochondrial lysate (~1 mg protein). The beads were then washed three times with PBS containing 0.1% Triton X-100. The immunoprecipitate was subsequently subjected to Western immunoblotting using calpastatin (or μ-calpain) antibody to assess co-immunoprecipatation with μ-calpain or calpastatin.

Determination of mitochondrial matrix free Ca^{2+} and assay of NCX activity

Loading of mitochondria with Fura-2. The isolated bovine pulmonary artery smooth muscle mitochondria were loaded with Fura-2 according to the method described by Cox and Matlib [24] with some modifications. Briefly, to the mitochondria (30 mg/ml), 10 μM Fura-2 AM was added and incubated for 10 min at 30 °C. This was then diluted with ice cold 180 mM KCl, 50 μM EGTA and then centrifuged at 8000*g* for 15 min to sediment the mitochondria. The sedimented mitochondria were resuspended in a small volume of the ice-cold medium to obtain a concentrated stock suspension of Fura-2 loaded mitochondria. The mitochondrial free Ca^{2+} was determined by measuring the Fura-2

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