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Mitochondrial calpain 10 is degraded by Lon protease after oxidant injury

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ABSTRACT

Calpain 10 is ubiquitously expressed and is one of four mitochondrial matrix proteases. We determined that over-expression or knock-down of mitochondrial calpain 10 results in cell death, demonstrating that mitochondrial calpain 10 is required for viability. Thus, we studied calpain 10 degradation in isolated mitochondrial matrix, mitochondria and in renal proximal tubular cells (RPTC) under control and toxic conditions. Using isolated renal cortical mitochondria and mitochondrial matrix, calpain 10 underwent rapid degradation at 37 °C that was blocked with Lon inhibitors but not by calpain or proteasome inhibitors. While exogenous Ca^{2+} addition, Ca^{2+} chelation or exogenous ATP addition had no effect on calpain 10 degradation, the oxidants tert-butyl hydroperoxide (TBHP) or H_2O_2 increased the rate of degradation. Using RPTC, mitochondrial and cytosolic calpain 10 increased in the presence of MG132 (Lon/proteasome inhibitor) but only cytosolic calpain 10 increased in the presence of epoxomicin (proteasome inhibitor). Furthermore, TBHP and H_2O_2 oxidized mitochondrial calpain 10, decreased mitochondrial, but not cytosolic calpain 10, and pretreatment with MG132 blocked TBHP-induced degradation of calpain 10. In summary, mitochondrial calpain 10 is selectively degraded by Lon protease under basal conditions and is enhanced under and oxidizing conditions, while cytosolic calpain 10 is degraded by the proteasome.

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Introduction

Fifteen mammalian calpains, Ca^{2+}-activated cysteine proteases, are divided into two groups: typical and atypical [1,2]. Typical calpains contain a Ca^{2+}-binding penta-EF hand in domain IV while atypical calpains do not. Calpains have been shown to be involved in many cellular processes such as: cytoskeletal/membrane rearrangements, signal transduction, cell cycle, and apoptosis. Limb girdle muscular dystrophy type 2A, gastric cancer, type II diabetes mellitus, Alzheimer's disease, myocardial infarcts, stroke, and acute kidney injury [3,4] have been linked to calpains.

Calpain 10 is an atypical calpain that is ubiquitously expressed and is localized to the cytosol, mitochondria and nucleus [5–7]. Our laboratory first reported calpain 10 being localized to rabbit kidney mitochondria [5]. Subsequently, mitochondrial calpain 10 has been found in rat and mouse kidney mitochondria [8]. While other laboratories have reported other calpains in the mitochondria [9,10], it is important to note that in our renal models we only detect calpain 10 [5]. Further research revealed that the mitochondrial matrix contains the majority of the mitochondrial calpain 10 activity and mitochondrial calpain 10 cleaves NDUFB8 and NDUFV2 (complex I proteins), ATP synthase β, and ORP150 (ER and mitochondrial chaperone) [5,11]. After Ca^{2+} overload, mitochondrial calpain 10 cleaves these substrates, which results in reduced state three respiration.

Interestingly, over-expression of calpain 10 induced mitochondrial swelling and cell death [5] and depletion of mitochondrial calpain 10 resulted in apoptosis [12]. These results provide evidence that maintaining homeostatic protein levels of mitochondrial calpain 10 is important for proper cellular function and viability. Much of the physiology and biochemistry of calpain 10 is unknown but it has been shown to be important for insulin secretion in pancreatic β cells and GLUT4-mediated transport in adipocytes and skeletal muscle [7,13]. In addition calpain 10 may play a role in renal aging. Renal calpain 10 decreased in aged rats, mice and humans [12] while calpain 10 protein levels did not change in the liver at any age and calpains 1 and 2 did not change in the kidney at any age.

Lon is an ATP-dependent protease that is important in protein quality control in the mitochondrial matrix [14,15]. Specifically, Lon degrades oxidized and misfolded proteins. In eukaryotes, it is a homo-oligomeric complex composed of seven monomers with a molecular weight of approximately 106 kDa [16,17]. Lon contains three domains: the N-terminal domain, the AAA+ domain, and P-domain. The N-terminal domain interacts with protein substrates [18]. The AAA+ domain contains two sub-domains: one is involved in ATP binding and the other is involved in ATP

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hydrolysis. The P-domain contains the active site (Ser/Lys dyad). There is no known consensus cleaving sequence for Lon, but it favors cleaving between hydrophobic amino acids [19,20]. Lon also degrades substrates linearly into peptides that are approximately 5–30 amino acids long [19–21].

As too much or too little calpain 10 activity results in cell death, we explored the mechanism of mitochondrial calpain 10 degradation.

Experimental

Reagents

Calpain 10 and Heat Shock Protein 60 (HSP60)[1] antibodies were purchased from Abcam (Calpain 10 product number – ab28226, Cambridge, MA). GAPDH and HRP-conjugated goat anti-rabbit/mouse secondary antibodies were obtained from Fitzgerald (Acton, MA) and Pierce (Rockford, IL), respectively. MG132, MG262, epoxomicin and calpeptin were purchased from Enzo Life Sciences (Plymouth Meeting, PA). Percoll was obtained from GE Healthcare (Piscataway, NJ). Trizol was purchased from Invitrogen (Carlsbad, CA). Reverse-transcriptase and SYBR Green Real-Time PCR kits were obtained from Fermentas Life Sciences (Glen Burnie, MD). Real-Time PCR primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). All other chemicals were obtained from Sigma (St. Louis, MO).

Mitochondrial isolation

Renal cortical mitochondria were isolated from female New Zealand White rabbits (2 kg) as described previously [5,22] and resuspended in mitochondrial isolation buffer (0.27 M sucrose, 5 mM Tris–HCl, and 1 mM EGTA pH 7.4) with or without 5 mM malate and 6 mM pyruvate. Mitochondria were further fractionated to isolate the mitochondrial matrix as previously described [5,23]. Briefly, mitochondria were purified on a Percoll/sucrose gradient followed by swelling of the outer mitochondrial membrane to isolate mitoplasts. Mitoplasts were sonicated briefly followed by centrifugation at 100,000*g* at 4 °C to obtain the inner mitochondrial membrane and the mitochondrial matrix.

Calpain 10 degradation assays

Whole mitochondria were incubated at 37 °C for various times followed by immunoblot analysis. In some experiments, whole mitochondria were pretreated with 10 mM EGTA, 10 μM calpeptin, 1:100 dilution of protease inhibitor cocktail (PI-Sigma), 10 μM CYGAbuK, 10 μM epoxomicin, 10 μM MG132 or 10 μM MG262 for 5 min prior to incubation at 37 °C. The Ca^{2+} experiments used 1.010 mM $CaCl_2$ to neutralize the 1 mM EGTA in the buffer, leaving 10 μM free Ca^{2+} followed by incubation at 37 °C. In other experiments, whole mitochondria were pre-treated with 1 μM antimycin A (AA), Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), 50 μM tert-butyl hydroperoxide (TBHP) or 100 μM H_2O_2 for 5 min prior to incubation at 37 °C for 15 or 30 min.

RPTC

Renal proximal tubules were isolated from female New Zealand White rabbits (2 kg) by the iron oxide perfusion method and grown on 35-mm dishes, in glucose-free media, until confluent (6 days) as described previously [24,25]. RPTC were treated with either 0.4 mM TBHP, 0.4 mM H_2O_2, 100 μM cisplatin, 1 μM antimycin A, or 0.5 μM FCCP for various times. At specific time points, cells were harvested and sonicated for 10 s in homogenization buffer (0.32 M sucrose, 50 mM Tris–HCl, 1 mM β-mercaptoethanol, 1 mM EDTA, pH 8.0) with protease inhibitor cocktail. Samples were centrifuged at 900*g* at 4 °C for 10 min, the supernatant was collected and centrifuged at 15000*g* at 4 °C for 10 min, to obtain the mitochondrial and cytosolic fractions [5,26].

Reverse-transcriptase reaction and Real-Time PCR

RPTC treated with TBHP, H_2O_2 or cisplatin were harvested in Trizol reagent (Invitrogen, Carlsbad, CA) and total RNA isolated following the manufacturer's instructions. RNA was quantified by measuring absorbance at 260 and 280 nm. To obtain cDNA, the reverse transcriptase kit (Fermentas, Glen Burnie, MD) and 1.5 μg of RNA was used according to the manufacturer's protocol. Real-Time PCR was performed on a Stratagene MX 3100P using 5 μL of a 1:5 dilution of cDNA added to 1x SYBR Green master mix and 400 nM primers. Primer sequences were: Calpain 10: sense – 5′-CACCT ACCTGCCGGACACA-3′ antisense – 5′-TGCCATGACGGAGACCTCTT-3′, α-Tubulin: sense – 5′-CTCTCTGTCGATTACGGCAAG antisense – 5′-TGGTGAGGATGGAGTTGTAGG.

Immunoblot analysis

Protein samples were separated by electrophoresis on a 4–12% SDS–PAGE gel prior to being transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked in a 2.5% non-fat milk/TBST (Tris-buffered saline Tween 20) solution for 1 h. All primary antibodies were incubated on a shaker overnight at 4 °C. Calpain 10, GAPDH and HSP60 antibodies were used at 1:1000 dilution and the HRP-conjugated secondary antibody (anti-rabbit or anti-mouse) was used at 1:100,000 dilution for 1 h at room temperature. An Alpha Innotech imaging system was used to visualize and quantify membranes for immunoreactive proteins using enhanced chemiluminescence detection.

Detection of oxidized mitochondrial calpain 10

Mitochondrial fractions were isolated from RPTC as described above and the Oxyblot kit (Millipore, Billerica, MA) was used as recommended by the manufacturer. Briefly, the carbonyl groups in the sample were derivatized with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone groups. The samples were separated by SDS–PAGE and immunoblot analysis conducted using an antibody directed against 2,4-dinitrophenylhydrazone.

Statistical analysis

A Student's *t*-test was performed to determine significance between two groups and a one-way ANOVA with a Student–Newman-Keuls test was used to determine significance between multiple groups. The sample size was at least three and a *p*-value $\leqslant 0.05$ required for statistical significance.

Results

Calpain 10 is degraded in whole mitochondria

To examine mitochondrial calpain 10 degradation in freshly isolated whole mitochondria, we incubated mitochondria at 37 °C for 30, 60 and 120 min. At 30 min there was a 53% decrease in calpain 10 protein levels (Fig. 1A). At 60 and 120 min, calpain 10 protein

[1] *Abbreviations used*: RPTC, renal proximal tubular cells; TBHP, tert-butyl hydroperoxide; HSP60, heat shock protein 60; AA, antimycin A; FCCP, Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; PI, protease inhibitor cocktail.