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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 Ca²⁺ addition, Ca²⁺ chelation or exogenous ATP addition had no effect on calpain 10 degradation, the oxidants tert-butyl hydroperoxide (TBHP) or H₂O₂ 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 H₂O₂ 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.

Keywords

Calpain 10; Lon; Renal Proximal Tubular Cells; Degradation; Toxicant; Inhibitors

INTRODUCTION

Fifteen mammalian calpains, Ca²⁺-activated cysteine proteases, are divided into two groups: typical and atypical [1-2]. Typical calpains contain a Ca²⁺-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

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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 NDUFB2 (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 3 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 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) 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 μ M calpeptin, 1:100 dilution of protease inhibitor cocktail (PI - Sigma), 10 μ M CYGAbuK, 10 μ M epoxomicin, 10 μ M MG132 or 10 μ M 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 μ M free Ca^{2+} followed by incubation at 37° C. In other experiments, whole mitochondria were pre-treated with 1 μ M antimycin A (AA), Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), 50 μ M tert-butyl hydroperoxide (TBHP) or 100 μ M 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 μ M cisplatin, 1 μ M antimycin A, or 0.5 μ M FCCP for various times. At specific time points, cells were harvested and sonicated for 10 sec 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 15,000 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 μ g of RNA was used according to the manufacturer's protocol. Real-Time PCR was performed on a Stratagene MX 3100P using 5 μ L 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'-CACCTACCTGCCGGACACA-3' antisense – 5'-TGCCATGACGGAGACCTCTT-3', α -Tubulin: sense – 5'-CTCTCTGTCTCGATTACGGCAAG antisense – 5'-TGGTGAGGATGGAGTTGTAGG.

Immunoblot Analysis

Protein samples were separated by electrophoresis on 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 hr. 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 hr 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 ≤ 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 (Figure 1A). At 60 and 120 min, calpain 10 protein levels decreased 68%. To determine whether calpain 10 degradation was altered in mitochondria with a membrane potential, metabolic substrates (5 mM malate/6 mM pyruvate) were added to the medium. Calpain 10 was equally degraded in the presence and absence of metabolic substrates (Figure 1A). Because calpain 10 is a Ca^{2+} -regulated protease we determined if exogenous Ca^{2+} or chelation altered degradation. Mitochondria with or without metabolic substrates were pretreated with 10 mM EGTA, a Ca^{2+} chelator, or 10 μM CaCl_2 and incubated at 37° C for 30 min. Pretreatment with EGTA or CaCl_2 did not affect the rate of calpain 10 degradation in the presence or absence of mitochondrial substrates (Figure 1B, data not shown). These results reveal that mitochondrial calpain 10 is readily degraded at 37° C in the presence and absence of a membrane potential and that Ca^{2+} does not play a role.

FCCP increases the rate of calpain 10 degradation

We next determined whether inhibition of the electron transport chain, using antimycin A, or dissipation of the proton gradient, using FCCP, altered calpain 10 degradation. While calpain 10 degradation trended to increase in mitochondria + substrates treated with 1 μM antimycin A, the increase was not statistically significant (Figure 1C). In contrast, calpain 10 degradation increased in the presence of 0.5 μM FCCP. Similar results were obtained in mitochondria treated with antimycin A and FCCP in the absence of metabolic substrates (data not shown). These data reveal that calpain 10 degradation is accelerated by dissipating the proton gradient.

Oxidant exposure increases calpain 10 degradation

Because Lon protease has been shown to preferentially cleave oxidized proteins [14], whole mitochondria were pretreated with 50 μM TBHP or 100 μM H_2O_2 prior to incubation at 37° C for 15 min. The 15 min control exhibited a 31% reduction in calpain 10 protein levels (Figure 1D). Whole mitochondria exposed to TBHP and H_2O_2 had ~65% reduction in

calpain 10. These results provide evidence that calpain 10 is degraded faster under oxidizing conditions.

Effect of protease inhibitors on mitochondrial calpain 10 degradation

Whole mitochondria were pretreated for 5 min with a protease inhibitor prior to being incubated at 37° C for 30 min in an attempt to block calpain 10 degradation. The protease inhibitors used were 10 μ M calpeptin, 10 μ M epoxomicin, 10 μ M CYGAbuK, 10 μ M MG132, 1 and 10 μ M MG262 and a 1:100 dilution of a protease inhibitor cocktail. Calpeptin is a cysteine protease inhibitor [27] and epoxomicin is a specific inhibitor for the proteasome [28]. Our laboratory developed a specific peptide inhibitor for calpain 10, CYGAbuK [29]. MG132 and MG262 are proteasome and Lon inhibitors, while MG132 also inhibits calpains [30]. The protease inhibitor cocktail contains 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin.

Figure 2A, reveals that calpain 10 decreased 58% in control mitochondria and that 10 μ M MG132, and 1 and 10 μ M MG262 blocked calpain 10 degradation. In contrast, in the presence of calpeptin, CYGAbuK and protease inhibitor cocktail calpain 10 degradation was similar to that observed in control mitochondria. Because four different calpain/cysteine inhibitors (calpeptin, CYGAbuK, leupeptin, E-64) did not block degradation, calpain 10 loss was not the result of calpains or other cysteine proteases. Likewise serine protease inhibitors, AEBSF, and aprotinin had no effect on calpain 10 degradation. Epoxomicin also had no effect on calpain 10 degradation, providing evidence that proteasome contamination is not responsible. Consequently, the decrease in calpain 10 degradation produced by MG132 and MG262 is due to Lon inhibition.

Calpain 10 degraded in mitochondrial matrix is not ATP dependent

Because the majority of mitochondrial calpain 10 activity is in the mitochondrial matrix [5], we confirmed the above results using mitochondrial matrix and determined ATP dependency. Mitochondrial matrix was pretreated with 10 μ M MG132 or diluent for 5 min prior to the addition of 165 μ M ATP or diluent. The samples were incubated at 37° C for 30 min. Calpain 10 protein levels decreased by 38% and this decrease was blocked by MG132 (Figure 2B). The decrease in calpain 10 levels was similar in the presence and absence of 165 or 5,000 μ M ATP (Figure 2B, data not shown). These experiments provide additional evidence that Lon degrades calpain 10 and that ATP does not stimulate degradation.

Cytosolic calpain 10 degradation

Since calpain 10 is found in the mitochondria and cytosol we determined if cytosolic calpain 10 undergoes rapid degradation as seen in mitochondria. Cytosol was incubated at 37° C for 30 min and immunoblotted for calpain 10. We found that cytosolic calpain 10 decreased ~20% when incubated at 37° C for 30 min (Figure 2C).

Mitochondrial calpain 10 decreases with TBHP treatment in RPTC

To determine if treatment with an oxidant causes a decrease in mitochondrial calpain 10, RPTC were treated with 0.4 mM TBHP for 5 min or 45 min. After 45 min of TBHP treatment, the media was removed and replaced with fresh media and RPTC were allowed to recover for 24 hr. This concentration of TBHP has been used previously in our laboratory and has been shown to be an effective method for inducing ROS damage in RPTC [31]. Bright field microscopic images of RPTC after 5 min of TBHP exposure revealed no damage, but at 45 min approximately 40% cell loss occurred and by 24 hr there was a slight recovery (Figure 3A). Mitochondrial calpain 10 protein levels did not change at 5 min, but at 45 min there was a 52% decrease in protein, compared to controls. This reduction in

mitochondrial calpain 10 protein remained at 24 hr (Figure 3B). In contrast, cytosolic calpain 10 protein did not change at 5 min, but increased 61% at 45 min and returned to control levels at 24 hr (Figure 3C).

Real-Time PCR was used to analyze calpain 10 mRNA levels. While, there was no change in the mRNA level at 5 min, at 45 min calpain 10 mRNA decreased 44% (Figure 3D). At 24 hr, calpain 10 mRNA levels trended to increase to 140% of control. The loss of mitochondrial calpain 10 after 45 min of TBHP exposure is consistent with our whole mitochondria experiments. These results illustrate that mitochondrial calpain 10 is selectively degraded in cells exposed to oxidants while cytosolic calpain 10 is not.

Protease inhibitor pretreatment causes calpain 10 accumulation

In Figure 2, MG132 was shown to decrease mitochondrial calpain 10 degradation in whole mitochondria and mitochondrial matrix. Therefore, we determined if MG132 decreases mitochondrial calpain 10 degradation in RPTC and if it could prevent TBHP-induced mitochondrial calpain 10 degradation. RPTC were pretreated with 10 μ M MG132 for 1 hr prior to TBHP or diluent exposure for 45 min. MG132 treatment caused a 1.6-fold accumulation of mitochondrial calpain 10 (Figure 4A). TBHP treatment alone decreased mitochondrial calpain 10 levels, confirming results in Figure 3B. When RPTC were treated with TBHP after MG132 pretreatment, calpain 10 degradation was diminished compared to RPTC treated with TBHP alone (Figure 4A). Cytosolic calpain 10 protein levels also accumulated with MG132 treatment (Figure 4B). This is likely because MG132 also inhibits the proteasome [30]. To confirm this possibility, we treated RPTC with 10 μ M epoxomicin for 1.75 hr and observed that cytosolic calpain 10 increased while mitochondrial calpain did not (Figure 4C). These experiments reveal that MG132 causes accumulation of mitochondrial calpain 10 under basal conditions and decreases TBHP-induced calpain 10 degradation, and that cytosolic calpain 10 degradation is blocked with epoxomicin.

Mitochondrial calpain 10 decreases with H₂O₂ treatment

To determine if another oxidant produces similar results to TBHP, we treated RPTC with 0.4 mM H₂O₂ for 1 and 24 hr. Figure 5A shows bright field microscopic images of RPTC treated with H₂O₂. At both times there was approximately 15% cell loss, a lesser degree of damage compared to TBHP treatment. Mitochondrial calpain 10 protein at 1 hr decreased 24% (Figure 5B) and returned to control levels at 24 hr. Cytosolic calpain 10 protein was unaffected at 1 or 24 hr with H₂O₂ treatment (Figure 5C). Real-Time PCR analysis of the calpain 10 mRNA revealed no change in mRNA levels at any time point (Figure 5D). These results reveal that limited oxidative stress selectively decreases mitochondrial calpain.

Mitochondrial calpain 10 is oxidized by TBHP and H₂O₂ treatment

To determine if mitochondrial calpain 10 is oxidized by TBHP and H₂O₂, we measured protein carbonyl groups using an Oxyblot kit (Millipore, Billerica, MA). RPTC were treated for 30 minutes with 400 μ M TBHP or H₂O₂ for 30 minutes, prior to significant mitochondrial calpain 10 degradation. A light staining band of 75 kDa corresponding to mitochondrial calpain 10 was observed on the oxyblot in mitochondria isolated from control RPTC (Figure 6). Following TBHP and H₂O₂ exposure strong staining bands on the oxyblot were observed at the 75 kDa. Quantification of these bands revealed a 3-fold increase in staining following oxidant exposure.

Mitochondrial calpain 10 in cisplatin toxicity

To determine whether a different cellular toxicant causes a decrease in mitochondrial calpain 10 protein levels we treated RPTC with 100 μ M cisplatin. Cisplatin is an anti-cancer

drug that causes nephrotoxicity and apoptosis, and we have previously characterized the toxicity of cisplatin in our RPTC model [32]. RPTC were treated with 100 μ M cisplatin for 1 hr and 24 hr. Bright field microscopic images show little cell death at 1 hr but by 24 hr there was approximately 30% apoptosis and cell loss (Figure 6A). Mitochondrial calpain 10 protein did not change at 1 hr but decreased 35% at 24 hr (Figure 6B). Cytosolic calpain 10 protein did not change at 1 or 24 hr (Figure 6C). Real-Time PCR revealed no change in calpain 10 mRNA at 1 hr but a 96% decrease at 24 hr (Figure 6D). These results provide additional evidence that mitochondrial calpain 10 is selectively lost in toxicant injury.

Mitochondrial respiration inhibitors and calpain 10

To determine whether inhibition of the electron transport chain (antimycin A) or dissipating the proton gradient with an uncoupler (FCCP) affects mitochondrial calpain 10, we treated RPTC with 1 μ M antimycin A and 0.5 μ M FCCP for 1 hr, time points that precede cell death. There was no change in mitochondrial or cytosolic calpain 10 levels in the presence or absence of FCCP or antimycin A (Figure 7).

DISCUSSION

Because we have shown that maintaining homeostatic levels of mitochondrial calpain 10 is important for cell viability [5, 12], mitochondrial calpain 10 degradation was examined under several conditions. In whole mitochondria and mitochondrial matrix, mitochondrial calpain 10 was rapidly degraded and Lon protease inhibitors, MG132 and MG262, prevented calpain 10 degradation. Additionally, treating mitochondria with oxidants (i.e. TBHP or H_2O_2) increased the rate of degradation. This is consistent with a previous report that Lon protease preferentially degrades oxidized proteins [14, 33]. Using RPTC, we demonstrated that the Lon protease inhibitor, MG132, causes an accumulation of mitochondrial calpain 10. TBHP and H_2O_2 rapidly oxidize and decrease mitochondrial calpain 10 and MG132 pretreatment blocks oxidant-induced loss of mitochondrial calpain 10. Taken together, mitochondrial calpain 10 is degraded by Lon protease under control conditions and is enhanced under oxidizing conditions. Additionally, using RPTC we show that epoxomicin-mediated proteasome inhibition caused accumulation of cytosolic calpain 10, defining the degradation pathway of cytosolic calpain 10.

While the exact physiological and pathological roles of mitochondrial calpain 10 have not been determined, it is known to decrease electron transport chain activity in the presence of elevated Ca^{2+} levels by hydrolyzing Complex I proteins (NDUFB8 and NDUFV2) and ATP synthase β . In contrast, loss of mitochondrial calpain 10 results in the accumulation of NDUFB8 and ATP synthase β . As such, calpain 10 is one of four mitochondrial matrix proteases. During oxidative stress, Lon-mediated degradation of calpain 10 may serve as a protective role with the goal of preserving mitochondrial proteins and function to facilitate mitochondrial and cellular repair. This is supported by previous reports that Lon preferentially degrades oxidized proteins [14, 33] and our observation that pretreatment of whole mitochondria and RPTC with TBHP and H_2O_2 resulted in increased degradation of mitochondrial calpain 10. Alternatively, the buildup of calpain 10 substrates may contribute to mitochondrial and cellular injury.

Using rabbit renal cortical mitochondria and mitochondrial matrix, we determined that cysteine proteases, calpains, and the proteasome were not responsible for mitochondrial calpain 10 degradation (Figure 2). In addition, a calpain 10 inhibitor and Ca^{2+} chelation had no effect on mitochondrial calpain 10 degradation; suggesting autolysis does not occur (Figures 2A and 1B). While the Lon protease inhibitors MG132 and MG262 blocked calpain 10 degradation, these inhibitors also have been reported to inhibit calpains (MG132 only), the proteasome and Lon [30]. Through the use of four other calpain/cysteine protease

inhibitors and one specific proteasome inhibitor, we conclude that MG132/262 prevents calpain 10 degradation by inhibiting Lon. Another soluble mitochondrial matrix protease, ClpP, is involved in protein quality control [34]. We do not believe that ClpP is involved in mitochondrial calpain 10 degradation because Haynes et al., showed that 50 μ M MG132 only inhibited approximately 50% of purified ClpP activity [35] and in our model we are able to completely inhibit mitochondrial calpain 10 degradation with 10 μ M MG132. While our attempts to confirm these data using Lon siRNA in our primary RPTC were unsuccessful because knock down of Lon could not be obtained, we think our use of Lon inhibitors are valid based on the above data and previously published articles [30, 36].

Since Lon is classified as an ATP-dependent protease we expected the addition of ATP would increase Lon activity, and more rapidly degrade mitochondrial calpain 10. However, ATP had no effect on the kinetics of calpain 10 degradation (Figure 2B). Bota and Davies characterized Lon degradation of aconitase and oxidized aconitase using purified protein and mitochondrial matrix [14]. Using purified protein, they showed that without ATP Lon degraded aconitase and oxidized aconitase but at 30% of the rate of the Lon + ATP group. Similar experiments were performed with mitochondrial matrix and the group without ATP degraded aconitase and oxidized aconitase at 20% and 50% of the rate of the mitochondrial matrix + ATP group, respectively. Therefore, under these conditions, ATP is not a requirement for Lon-mediated calpain 10 degradation.

Because Lon degraded mitochondrial calpain 10 in our whole mitochondria experiments, we wanted to determine if Lon was responsible for mitochondrial calpain 10 hydrolysis in RPTC under control conditions and following oxidant exposure. Mitochondrial fractions isolated from RPTC treated with MG132 exhibited a marked increase in calpain 10 (Figure 4A). Treating RPTC with TBHP caused a rapid decrease in mitochondrial calpain 10 by 45 min and this decrease remained at 24 hr (Figure 3B). RPTC pretreated with MG132 for 1 hr prior to TBHP exposure for 45 min resulted in less mitochondrial calpain 10 degradation (Figure 4A), revealing that Lon degrades mitochondrial calpain 10 under control and oxidizing conditions in cells.

In contrast, cytosolic calpain 10 from RPTC treated with TBHP exhibited increased protein levels of approximately 60% at 45 min, before returning to control levels at 24 hr (Figure 3C). This was unexpected, but shows that cytosolic calpain 10 is not targeted for degradation following oxidative stress. In addition, we propose that the decrease in mitochondrial calpain 10 when cytosolic calpain 10 is increased is due to inhibition of mitochondrial import of cytosolic calpain 10. There has been at least one report that oxidative stress inhibits mitochondrial protein import [37]. While we have previously reported that the first 15 amino acids on the N-terminus is responsible for mitochondrial import [5], the regulation of this import under control and oxidative stress is not known. Alternatively, the increase in cytosolic calpain 10 could be the result of oxidant-induced proteasome inhibition. There have been several reports of oxidant-induced proteasome inhibition [38-42]. It is possible that mitochondrial calpain 10 is being exported to the cytosol after TBHP treatment, but we do not believe this is occurring because of two reasons. H_2O_2 causes a decrease in mitochondrial calpain 10, albeit to a lesser extent than TBHP, but has no effect on cytosolic calpain 10 protein or mRNA. If oxidant stress was causing an exportation of mitochondrial calpain 10 then why does H_2O_2 not cause a similar effect. The other reason is that the amount of cytosolic calpain 10 is much greater than mitochondrial calpain 10. We have shown how knockdown of calpain 10 results in reduction of the mitochondrial form much quicker than the cytosolic form [12, 43]. Thus, there is always a gradient from the cytosol to the mitochondria, so it is unlikely that there is ever a reversal of this gradient [43].

Mitochondrial calpain 10 was decreased by TBHP, H₂O₂ and cisplatin in the absence of changes in cytosolic calpain 10 and whether calpain 10 mRNA decreased or not. The main similarity between these toxicants is that they produce oxidative stress. In contrast, the specific mitochondrial toxicants antimycin A and FCCP did not alter mitochondrial or cytosolic calpain 10 protein levels in RPTC (Figure 7).

Little is known concerning the degradation of cytosolic calpain 10. Using cytosol, calpain 10 was degraded but not as rapidly as in mitochondria (Figure 2C). Since epoxomicin is a specific inhibitor of the proteasome [28], we treated RPTC with epoxomicin and showed that cytosolic calpain 10 accumulated (Figure 4C), providing evidence that cytosolic calpain 10 is degraded by the proteasome.

The observation that calpain 10 was rapidly degraded in whole renal cortical mitochondria or mitochondrial matrix at 37° C was surprising but consistent. Since we have previously shown that mitochondria isolated by these methods are healthy and have high ADP-stimulated oxygen consumption [5], we do not propose the rapid degradation is the result of damaged mitochondria. Furthermore, mitochondrial calpain 10 was rapidly degraded in RPTC mitochondria exposed to oxidants. We propose that mitochondrial calpain 10 is susceptible to oxidation, particularly from ROS produced in the mitochondrial matrix and that oxidation may be a primary mechanism by which mitochondrial calpain 10 is regulated. However, it is possible that a protective/regulatory mitochondrial calpain 10 binding protein is lost under these *in vitro* conditions. In contrast, even though cytosolic calpain 10 and mitochondrial calpain 10 are thought to be equivalent, cytosolic calpain 10 was not readily degraded by the proteasome in the cytosol under control and oxidizing conditions. There are a number of reasons why these differences exist and include 1) different oxidative environments, 2) different mechanisms of degradation, and 3) different protective/regulatory associated proteins.

In summary, mitochondrial calpain 10 is selectively degraded by Lon protease under basal conditions and is enhanced under oxidizing conditions while cytosolic calpain is degraded by the proteasome. Understanding calpain 10 regulation is important, considering calpain 10 needs to be maintained within a specific level to maintain renal cell health.

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Abbreviations

RPTC	renal proximal tubular cells
TBHP	tert-butyl hydroperoxide
HSP60	heat shock protein 60
AA	antimycin A
FCCP	Carbonyl cyanide- <i>p</i> -trifluoromethoxyphenylhydrazone
PI	protease inhibitor cocktail

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Highlights

- Heat and oxidant stress induce mitochondrial calpain 10 degradation
- Lon inhibitors are able to prevent mitochondrial calpain 10 degradation
- Epoxomicin, a proteasome inhibitor, causes an accumulation in cytosolic calpain 10

Figure 1.

Calpain 10 degradation in whole mitochondria. *A*, Incubation of whole mitochondria at 37°C for 30, 60 and 120 min induced calpain 10 degradation in the absence and presence of metabolic substrates (pyruvate/malate, Subs). *B*, Mitochondria pretreated (5 min) with 10 mM EGTA or 10 μM CaCl₂ had no effect on calpain 10 degradation after 30 min. *C*, Mitochondria pretreated (5 min) with 1 μM antimycin A had no effect on calpain 10 degradation while calpain 10 degradation increased in the presence of 0.5 μM FCCP at 30 min. *D*, Pretreatment of mitochondria with 50 μM TBHP or 100 μM H₂O₂ for 5 min increased calpain 10 degradation at 15 min. All pretreatments were on ice. Immunoblot analysis was performed on calpain 10 (75 kDa) and quantified by densitometric analysis. Data are represented as means ± SEM of % of control (N ≥ 3 for each group). Bars with different superscripts are statistically different from one another (p ≤ 0.05).

Figure 2.

Effect of protease inhibitors on mitochondrial calpain 10 degradation. **A**, Mitochondria pretreated (5 min) with 10 μ M MG132, 1 or 10 μ M MG262 blocked mitochondrial calpain 10 degradation after 30 min at 37° C. **B**, Mitochondrial matrix pretreated with 165 μ M ATP or diluent had no effect on calpain 10 degradation. Mitochondrial matrix pretreated (5 min) with 10 μ M MG132 blocked calpain 10 degradation after 30 min at 37° C. **C**, Cytosolic calpain 10 is degraded slower than mitochondrial calpain 10. All pretreatments were on ice. Immunoblot analysis was performed on calpain 10 (75 kDa). HSP60 (60 kDa) and GAPDH (37 kDa) served as loading controls and quantified by densitometric analysis. Data are represented as means $\pm$ SEM of % of control ($N \geq 3$ for each group). Bars with different superscripts are statistically different from one another ($p \leq 0.05$). Bars with * are statistically different from controls ($p \leq 0.05$).

Figure 3.

TBHP induces degradation of mitochondrial calpain 10 in RPTC. RPTC were treated with 0.4 mM TBHP for 5 min, 45 min and 24 hr, and RNA, and mitochondrial and cytosolic fractions harvested. *A*, Brightfield photomicrographs (100x magnification). *B*, Immunoblot analysis of mitochondrial calpain 10 (75 kDa). *C*, Immunoblot analysis of cytosolic calpain 10 (75 kDa). HSP60 (60 kDa) and GAPDH (37 kDa) served as loading controls and quantified by densitometric analysis. *D*, Calpain 10 mRNA levels were determined by Real-Time PCR with α -tubulin serving as a loading control. Data are represented as means $\pm$ SEM of % of control ($N \geq 3$ for each group). Bars with * are statistically different from controls ($p \leq 0.05$).

Figure 4.

Protease inhibitor pretreatment causes calpain 10 accumulation in RPTC. RPTC were pretreated with 10 μ M MG132 for 1 h followed by 0.4 mM TBHP treatment for 45 min and mitochondrial and cytosolic fractions were harvested. *A*, Immunoblot analysis of mitochondrial calpain 10 (75 kDa). *B*, Immunoblot analysis of cytosolic calpain 10 (75 kDa). *C*, RPTC were treated with 10 μ M epoxomicin for 1.75 hr and mitochondrial and cytosolic fractions were harvested. Immunoblot analysis of mitochondrial and cytosolic calpain 10 (75 kDa). HSP60 (60 kDa) and GAPDH (37 kDa) served as loading controls and quantified by densitometric analysis. Data are represented as means $\pm$ SEM of % of control ($N \geq 3$ for each group). Bars with different superscripts are statistically different from one another ($p \leq 0.05$). Bars with * are statistically different from controls ($p \leq 0.05$).

Figure 5.

H₂O₂ induces rapid degradation of mitochondrial calpain 10 in RPTC. RPTC were treated with 0.4 mM H₂O₂ for 1 and 24 h and RNA, and mitochondrial and cytosolic fractions harvested. *A*, Brightfield photomicrographs (100x magnification). *B*, Immunoblot analysis of mitochondrial calpain 10 (75 kDa). *C*, Immunoblot analysis of cytosolic calpain 10 (75 kDa). HSP60 (60 kDa) and GAPDH (37 kDa) served as loading controls and quantified by densitometric analysis. *D*, Calpain 10 mRNA levels were determined by Real-Time PCR with α -tubulin serving as a loading control. Data are represented as means $\pm$ SEM of % of control (N $\geq$ 3 for each group). Bars with * are statistically different from controls ($p \leq 0.05$).

Figure 6.

Detection of oxidized calpain 10. RPTC were treated with 400 μ M TBHP or H_2O_2 30 min and the mitochondrial fraction was harvested. Immunoblot analysis of 2,4-dinitrophenylhydrazine and calpain 10. The calpain 10 antibody detects a band that is slightly below 75 kDa. HSP60 (60 kDa) served as loading controls and quantified by densitometric analysis. Data are represented as means $\pm$ SEM of % of control ($N \geq 3$ for each group). Bars with different superscripts are statistically different from one another ($p \leq 0.05$).

Figure 7.

Mitochondrial calpain 10 in cisplatin toxicity. RPTC were treated with 100 μ M cisplatin for 1 and 24 hr and RNA, and mitochondrial and cystolic fractions harvested. *A*, Brightfield photomicrographs (100x magnification). *B*, Immunoblot analysis of mitochondrial calpain 10 (75 kDa). *C*, Immunoblot analysis of cytosolic calpain 10 (75 kDa). HSP60 (60 kDa) and GAPDH (37 kDa) served as loading controls and quantified by densitometric analysis. *D*, Calpain 10 mRNA levels were determined by Real-Time PCR with α -tubulin serving as a loading control. Data are represented as means $\pm$ SEM of % of control ($N \geq 3$ for each group). Bars with * are statistically different from controls ($p \leq 0.05$).

Figure 8. Mitochondrial respiration inhibitors and calpain 10. RPTC were treated with 1 μ M antimycin A or 0.5 μ M for 1 hr and mitochondrial and cytosolic fractions harvested. Immunoblot analysis of mitochondrial and cytosolic calpain 10 (75 kDa), HSP60 (60 kDa) and GAPDH (37 kDa) served as loading controls and quantified by densitometric analysis. Data are represented as means $\pm$ SEM of % of control ($N \geq 3$ for each group). Bars with * are statistically different from controls ($p \leq 0.05$).