Cardiomyocyte Degeneration With Calpain Deficiency Reveals a Critical Role in Protein Homeostasis

Anita S. Galvez, Abhinav Diwan, Amy M. Odley, Harvey S. Hahn, Hanna Osinska, Jaime G. Melendez, Jeffrey Robbins, Roy A. Lynch, Yehia Marreez, Gerald W. Dorn II

Abstract—Regulating the balance between synthesis and proteasomal degradation of cellular proteins is essential for tissue growth and maintenance, but the critical pathways regulating protein ubiquitination and degradation are incompletely defined. Although participation of calpain calcium-activated proteases in post–necrotic myocardial autolysis is well characterized, their importance in homeostatic turnover of normal cardiac tissue is controversial. Hence, we evaluated the consequences of physiologic calpain (calcium-activated protease) activity in cultured cardiomyocytes and unstressed mouse hearts. Comparison of in vitro proteolytic activities of cardiac-expressed calpains 1 and 2 revealed calpain 1, but not calpain 2, activity at physiological calcium concentrations. Physiological calpain 1 activation was evident in adenoviral transfected cultured cardiomyocytes as proteolysis of specific substrates, generally increased protein ubiquitination, and accelerated protein turnover, that were each inhibited by coexpression of the inhibitor protein calpastatin. Conditional forced expression of calpain 1, but not calpain 2, in mouse hearts demonstrated substrate-specific proteolytic activity under basal conditions, with hyperubiquitination of cardiac proteins and increased 26S proteasome activity. Loss of myocardial calpain activity by forced expression of calpastatin diminished ubiquitination of 1 or more specific myocardial proteins, without affecting overall ubiquitination or proteasome activity, and resulted in a progressive dilated cardiomyopathy characterized by accumulation of intracellular protein aggregates, formation of autophagosomes, and degeneration of sarcomeres. Thus, calpain 1 is upstream of, and necessary for, ubiquitination and proteasomal degradation of a subset of myocardial proteins whose abnormal accumulation produces autophagosomes and degeneration of cardiomyocytes with functional decompensation. (Circ Res. 2007;100:1071-1078.)

Key Words: metabolism ■ protease ■ ubiquitin

B"eating of the heart for a lifetime requires constant renewal of the molecular contractile machinery through a process of regulated protein turnover. Although much has been learned about the transcriptional and translational regulation of cardiac proteins,1 the opposing functions controlling protein degradation are poorly understood. Clearly, protein degradation and formation must be balanced to maintain cellular homeostasis, and an imbalance is likely to result in cellular dysfunction and disease.

Because wasting and dystrophic diseases of skeletal muscle have been intensively investigated, more is known about protein turnover in skeletal than cardiac muscle. Two proteolytic systems are recognized, the proteasome and calpains. The proteasome is a ubiquitin-dependent proteolytic system capable of degrading intact monomeric myofibrillar proteins, but not high-molecular-weight-protein complexes.2,3 This suggests that an additional proteolytic system upstream of the ubiquitin/proteasome system disassembles multiprotein complexes of the sarcomere, and although the idea remains controversial, skeletal muscle–specific calpain 3 has been suggested to perform this function.4,5 Accordingly, loss-of-function mutations of this protease result in a unique form of muscular dystrophy, limb girdle muscular dystrophy 2A (LGMD2A).6,7

As in skeletal muscle, the ubiquitin/proteasome system plays an important role in cardiac protein degradation.8 Unlike skeletal muscle, however, there is no heart-specific calpain that might facilitate this process by targeted proteolysis of macromolecular protein complexes. Instead, myocardium contains modest amounts of ubiquitous calpains 1 and 2 that have been studied largely as mediators of postischemic cardiac injury.9–11 There are no data addressing the notion that calpains 1 or 2 may have an essential physiological function in myocardium. Nevertheless, it is important to determine whether this might be the case, not only for the mechanistic insight to be gained, but because pharmacological calpain inhibition is being pursued as a means of limiting cardiac ischemic injury.11,12 Here, to identify the conse-
quences of calpain activity on myocardial protein degradation and function, we used genetic techniques to positively and negatively modulate calpain activity in cultured cardiac myocytes and in vivo mouse hearts. The results suggest that calpain 1 can promote ubiquitination of myocardial proteins and is positioned at the apex of an essential degradative pathway that prevents pathological accumulation of select misfolded or damaged proteins. Suppression of cardiac calpain activity by calpastatin overexpression inhibits ubiquitination of some proteins, resulting in formation of intracellular protein aggregates, stimulation of cardiomyocyte autophagy, and cardiomyopathic degeneration.

Materials and Methods

Calpain Activity
Casein zymogram assays were modified from Croall et al. Myocardial protein (300 to 600 μg per lane) was size separated in 8% nondenaturing polyacrylamide gels containing 0.5% casein sodium salt. Calpain activity was developed overnight at room temperature in 25 mmol/L 4-morpholinopropanesulfonic acid (pH 7.5), 5 mmol/L 2-mercaptoethanol, and either 5 μmol/L or 5 mmol/L CaCl2 (for calpain 1 and total calpain, respectively), or an intermediate concentration as specified; protein samples were then fixed and stained with Coomassie blue. Calpain protease activity assay was modified from Ishiura et al. Protein samples were incubated for 30 minutes at 30°C with 0.24% casein Hammerstein grade, 25 mmol/L 2-mercaptoethanol, 0.1% imidazol (pH 7.5), and either 5 μmol/L or 5 mmol/L CaCl2, and assayed by absorbance at 280 nm. Calpain protein standards (calpain 1 from porcine erythrocytes and rat recombinant calpain 2; Calbiochem) were assayed simultaneously to generate concentration-activity curves.

Calpain-1 and Calpastatin Adenovirus Generation
The calpain 1 and calpastatin adenoviruses were generated with the AdEasy XL adenoviral vector system (Stratagene, La Jolla, Calif) using a 2637-bp mouse calpastatin cDNA (GenBank accession no. BF540707) or a 3077 bp mouse calpain 1 cDNA (GenBank accession no. NM007600). Recombinant virus were plaque purified, expanded, and titered by duplicate plaque assays in agarose gel overlays of HEK293 cells.

Conditional Calpain and α-Myosin Heavy Chain Calpastatin Transgenic Mice
Animal studies were performed in accordance with University of Cincinnati animal care and use protocols, as outlined by the International Animal Care and Use Committee. Conditional cardiac-specific calpain 1, calpain 2 (GenBank accession no. NM009794), and calpain 3 (GenBank accession no. NM007601) transgenic mice were generated by subcloning the respective mouse cDNAs into the SalI–HindIII site of pTEToff α-myosin heavy chain (MHC) in the tetracycline-suppressible binary α-MHC promoter transgene system. Mice were studied under the following conditions: (1) mice never received doxycycline, and the transgene was expressed during neontal period; (2) pregnant dams, suckling dams, and weaned pups received doxycycline (0.2 mg/mL in drinking water with 2% sucrose) until 8 weeks of age, after which time, it was withdrawn (adult induction). Control mice had continued doxycycline suppression. A calpastatin transgenic mouse was generated by subcloning the mouse calpastatin cDNA into the SalI/HindIII site of the conventional cardiac α-myosin heavy chain promoter.

Proteasome Activity Assay
The activity of proteasomes was assayed in 10 μg of ventricular homogenates incubated with the fluorogenic substrate Suc-LLVY-AMC using the Chemicon International 20S Proteasome Activity Assay Kit. The 20S proteasome is the catalytic subunit of the 26S proteasome. All measurements were performed in triplicate and replicated in independent experiments. Coincubation of reactants with the specific proteasome inhibitor lactastatin decreased measured proteasome activity by more than 90% in all experiments.

Functional Assessments
Two-dimensional guided M-mode echocardiography of unsedated mice measured left ventricular diastolic and systolic dimensions (left ventricular end-diastolic dimension and left ventricular end-systolic dimension), from which fractional shortening was derived. Pulsed-wave Doppler was used to measure aortic ejection time and calculate velocity of circumferential shortening (fractional shortening/ejection time). Invasive hemodynamic studies were performed on anesthetized, spontaneously breathing 8- to 12-week-old transgenic mice and their nontransgenic (NTG) littermate controls using 1.4 French Millar solid-state catheters. Cell culture, immunoblot, and immunohistochemical methodological details are provided in the online data supplement, available at http://circres.ahajournals.org.

Statistical Analysis
Results are mean±SE. Experimental groups were compared using Student’s t test or 1-way ANOVA. A Bonferroni test was used for post hoc comparisons, with P<0.05 indicating significance.

Results

Modulation of Calpain Activity in Cultured Cardiomyocytes
We used casein zymography to compare the calcium sensitivities of purified calpains 1 and 2. Calpain 1 was fully active in 0.5 μmol/L calcium, but there was no detectable calpain 2 activity at concentrations lower than 50 μmol/L (Figure 1a). These results indicate that calpain 1 has more proteolytic activity than calpain 2 at calcium concentrations that are present in cardiac myocytes.

Calpains can be upregulated and/or activated in cultured cardiac myocytes by physiological stresses that increase intracellular calcium concentrations, such as angiotensin II and hypoxia. Here, we used adenoviral expression of calpain 1 or its inhibitor protein, calpastatin, to positively and negatively modulate calpain activity in cultured neonatal rat cardiomyocytes and determine whether calpain 1 has measurable proteolytic activity in nonstressed cardiomyocytes. Calpastatin is a highly specific inhibitor of calpain 1 and calpain 2 that does not inhibit the activity of any other protease tested, including papain, cathepsin B, bromelin, ficin, trypsin, chymotrypsin, plasmin, thrombin, pepsin, calpains, or thermolysin. Thus, calpastatin expression is an effective means of globally inhibiting cellular calpain 1 and calpain 2 activity.

Cultured cardiac myocytes infected with adenocalpastatin (not shown) or adenocalpain 1 (Figure 1b and 1c) exhibited normal morphology and cytoskeletal integrity after 24 hours with no increase in apoptotic or necrotic cell death (Figure 1c). However, after 72 hours, cardiomyocyte death was widespread (not shown). A strong immunoblot signal for calpain 1 was not detected in cells, despite evidence for calpain-specific proteolysis of known endogenous substrates (Figure 1d), suggesting that high calpain 1 expression in nonstressed cells may result in significant proteolytic activity that is sufficient to cause progressive cellular dysfunction (suicide transgene). Indeed, adenocalpain 1 infection resulted in characteristic fragmentation of three calpain sub-
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strates, desmin, protein kinase Ca, and troponin I (Figure 1d) into their calpain cleavage products.10,27–29 However, consistent with absence of cell death or TUNEL labeling at this time point, proteolysis of Bcl-2 family proteins, including the known cardiomyocyte calpain substrate, Bid,30 was not observed (Figure 1d). Together, these results demonstrate significant, but selective, calpain 1 activity in nonstressed cultured cardiac myocytes.

Calpain Involvement in Cardiomyocyte Protein Turnover

The possible consequences of calpain activity on protein turnover in nonstressed cardiomyocytes were interrogated by labeling the protein pool with ³H-tyrosine. Adeno-calpain 1 increased tyrosine release into the culture medium in a time-dependent manner over 24 hours, which could be inhibited by coinfection with adeno-calpastatin (Figure 1e).

These results show that calpain 1 accelerates protein degradation in cultured cardiomyocytes. To determine whether the ubiquitin/proteasome degradation pathway was involved, extracts from cardiac myocytes infected with adeno-calpain 1 or adeno-calpastatin were examined for ubiquitinated protein conjugates. Calpain 1 transfection caused the accumulation of ubiquitinated protein conjugates, which was inhibited by coexpression of calpastatin (Figure 1f). These data show that calpain 1 can facilitate ubiquitination of cardiac myocyte proteins destined for proteasomal degradation.

Genetic Modulation of Myocardial Calpains in Transgenic Mice

As our tissue culture studies indicated that calpain 1 can influence cardiomyocyte protein ubiquitination and turnover, we reasoned that calpain activity might play a role in cardiac remodeling. To test this notion required modulation of calpain activity in vivo. Preliminary attempts to express calpains 1 and 2 in the heart using conventional transgenic techniques resulted in no founder mice, suggesting an embryonic lethal effect of unregulated myocardial calpain activity. Therefore, we used a bitransgenic conditional cardiac expression system16 to generate viable mouse lines with myocardial overexpression of calpains. Conventional cardiac transgenesis18 was used to inhibit myocardial calpain activity by overexpressing calpastatin. Myocardial calpain expression was then assessed as maximum calcium-stimulatable enzymatic activity using spectrophotometry14 and casein zymography.13 (Note: these studies use the activity assay to assess enzyme content and do not reflect intrinsic calpain activity.)

In control hearts, the proportion of calpain activity attributable to calpain 1 (ie, calpain activity at 5 μmol/L Ca²⁺) was less than 20% of total Ca²⁺-stimulated myocardial calpain activity (Figure 2a, right, and Figure 2b, NTG). Of 3 calpain 1 transgenic mouse lines, the line with the greatest calpain 1 activity (L3) more than doubled cardiac calpain 1–specific activity (Figure 2a, left, and Figure 2b, L3). Calpain 2 was not affected by calpain 1 overexpression (Figure 2b). In adult calpastatin transgenic mice, the spectrophotometric activity assay showed that maximum cardiac calpain 1–specific activity was decreased from 3.3±0.2 to 1.4±0.1 U/mg (58% inhibition, n=3 pairs, P<0.01) and that overall calpain activity was decreased from 19.9±0.1 to 13.3±0.9 U/mg (34% inhibition, n=3 pairs, P<0.01), which was confirmed by casein zymography using purified calpains (Figure 2c). The zymography studies also showed that calpain 2 has less casein proteolytic activity than calpain 1 at comparable calcium levels (Figure 2c, NTG). These results demonstrate that forced cardiac expression of calpain 1 or calpastatin can be used to modulate myocardial calpain activity.

Substrate Specificity in Conditional Calpain 1—Overexpressing Mouse Hearts

The 3 lines of calpain 1 transgenic mice (L1, L2, L3, in ascending order of calpain 1 activity) were characterized after...
neonatal (≈1 week) or adult (8 weeks of age) calpain induction. Consistent with our findings in cultured cells that calpain 1 has some activity in the basal state, and recapitulating the previously postulated role for calpain activation in postischemic myocardial stunning and cardiac decompensation,10–12 calpain 1 induction within the first 2 weeks of life caused early mortality in the 2 higher-expressing lines (Figure 3a, left), with life spans inversely proportional to calpain 1 activity. In adult hearts, heart failure developing only in line L3 (Figure 3b, left). In all cases, lethality was associated with cardiac enlargement (Figure 3a and 3b, right), ventricular dilation, and diminished ejection performance (Table). Histological examination of failing L3 hearts revealed myocyte necrosis and a generalized mononuclear cell infiltrate (Figure 3c). In 2-week-old L3, L2, and L1 mice with neonatal induction of calpain 1, fragmentation of desmin and protein kinase Cα (but not troponin I) into characteristic calpain proteolysis products27,28 (Figure 3d) and ubiquitination of myocardial proteins (Figure 3e) correlated with calpain activity. Notably, increased proteasomal activity in a range similar to that previously described in chronic pressure overload hypertrophy31 and increased ubiquitination were seen in adult-expressing L2 mice, which do not develop significant pathology. These results show that calpain 1 can exhibit physiologically significant enzymatic activity in normal, unstressed myocardium, increasing ubiquitination of cardiac proteins and proteasomal activity. The data also reveal increased sensitivity to high levels of calpain 1 activity in neonatal hearts, resulting in cardiac myocyte necrosis and cardiomyopathy that resemble some well-established pathological effects of calpains in stressed myocardium.26,32

The results in cultured cardiac myocytes and transgenic mouse hearts showed that calpain 1 is less abundant, but has greater calcium sensitivity and proteolytic activity under basal conditions, than calpain 2 (see Figure 2). This suggested that calpain 2 is poorly suited to regulate myocardial prote-
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Cardiac Calpainopathy With Calpastatin Overexpression

To further explore whether calpains have an essential function in the normal heart, we inhibited cardiac myocyte calpain activity by forced expression of calpastatin (Figure 4). Calpastatin overexpression at levels that inhibited myocardial calpain 1 activity by 58% (see also Figure 2c) resulted in a slowly progressive dilated cardiomyopathy. Ventricular ejection performance, measured as percentage of fractional shortening, was decreased (Table and Figure 4a, bottom) and responsiveness to β-adrenergic stimulation was diminished (Figure 4b and supplemental Table II). The integrity of myocardial calpain target proteins was maintained and there was no counter regulation of other cardiac calpains (Figure 4c), but expression of pathological cardiac genes was increased (Figure 4d). In approximately half of the calpastatin mice, atrial arrhythmias occurred (supplemental Figure III). Histological examination of calpain-overexpressing myocardium showed features similar to skeletal muscle biopsies in patients with LGMD2A, including variability of myocyte size (Figure 4a and 4e), frequent intracellular inclusions (Figure 4a, top), and distorted myofibrillar architecture with regionally abnormal or absent staining for sarcolemmal dystrophin (Figure 4a, middle). Mean cardiomyocyte cross-sectional area was 332±11 μm² in calpastatin versus 252±22 μm² in NTG (n = 500 to 600 cells from each of 3 pairs of hearts, P < 0.05).

Transmission electron microscopy of calpastatin transgenic hearts revealed an ultrastructural picture that is atypical for mouse models of heart failure, with extensive sarcomeric disruption, formation of amorphous protein aggregates, frequent myelin bodies, and abundant autophagic vacuoles (Figure 5a through 5f). Because calpain 1 increased protein ubiquitination in cardiac myocytes and transgenic mouse hearts, which could be inhibited by calpastatin in cultured neonatal cardiac myocytes, we considered that myocardial olysis under normal physiological conditions. To test this notion, the bitransgenic inducible system was used to over-express calpain 2 in the heart, which resulted in no functional or biochemical phenotype (Figure I and Table I in the online data supplement). Thus, under normal conditions or when overexpressed, calpain 2 does not exhibit enzymatic activity in the normal heart. To control for the possibility that an unanticipated or unique characteristic of calpain 1 accounted for its effects in the heart, we also ectopically expressed a calpastatin-insensitive calpain, skeletal muscle–specific calpain 3, in the heart. Myocardial expression of calpain 3 reproduced the phenotype seen with higher level calpain 1 overexpression (supplemental Figure II and supplemental Table I). These results show that cardiac-expressed calpain 1 and skeletal muscle calpain 3 have similar activities under basal conditions. Because loss-of-function mutations of calpain 3 are known to cause human limb girdle muscular dystrophy, this finding suggested that inhibition of cardiac calpain 1 might be equally important to cardiac health.

Table I. These results show that cardiac-expressed calpain 1 and skeletal muscle calpain 3 have similar activities under basal conditions. Because loss-of-function mutations of calpain 3 are known to cause human limb girdle muscular dystrophy, this finding suggested that inhibition of cardiac calpain 1 might be equally important to cardiac health.

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calpain inhibition by calpastatin might cause a pathological accumulation of nonubiquitinated proteins. Indeed, whereas immunoblot analysis showed no change in overall ubiquitin content in calpastatin hearts, specific high-molecular-weight-protein bands showed diminished ubiquitin content (Figure 5g). Because overall proteasomal activity was not increased in the calpastatin transgenic hearts (Figure 5g, right), these results suggest a calpain effect on normal protein ubiquitination, but not directly on the 26S proteasome. Taken together, we interpret these findings to indicate that myocardial calpain activity is necessary for normal ubiquitination and ubiquitin-dependent proteasomal degradation of specific, but as yet unidentified high-molecular-weight cardiac proteins, but not for many other cardiac proteins that are degraded through the proteasome pathway (Figure 6). Chronic inhibition of basal calpain activity likely results in the observed accumulation and aggregation of nonubiquitinated misfolded proteins intended for proteasomal degradation that are shunted to, and overwhelm, autophagic destruction pathways, ultimately causing a dilated cardiomyopathy characterized by degeneration and oncosis of cardiac myocytes.

**Discussion**

The role of protein turnover in myocardial homeostasis can be overlooked when the focus is mainly on molecular determinants of gene expression. Balanced protein turnover is essential for tissue maintenance, whereas properly modulated protein turnover is a prerequisite for growth. The primary nonlysosomal protein degradative pathway is the ubiquitin/proteasome system, which destroys damaged or senescent proteins and provides for renewal of cellular infrastructure by newly synthesized protein. When something interferes with normal protein degradation, misfolded or damaged proteins accumulate in insoluble inclusions and protein aggregates that ultimately lead to oncotic cell death.

In the heart and other striated muscle, the ubiquitin/proteasome is the major pathway for homeostatic protein breakdown. However, because the proteasome cannot degrade intact myofibrils, a mechanism must exist to disassemble macromolecular myofibrillar complexes before ubiquitination and proteasomal degradation of their constituent proteins. We propose that myocardial calpains can serve this function. Although the precise identity of myocardial (current report) or skeletal muscle proteins for which calpain activity is essential to ubiquitination is not known, our results show that calpain activity is necessary and sufficient for ubiquitination of specific proteins in the heart and that it is can be a critical sensor for, and effector of, myocardial protein degradation. Our results are consistent with the notion that failure of calpain-dependent processes in the ubiquitin/proteasome pathway cause aggregation of nondegraded protein, triggering the alternate disposal pathway of autophagy. Indeed, autophagic vacuoles and protein aggregates were the characteristic features of degenerated cardiomyocytes in calpastatin hearts, and prior studies have shown that autophagy is induced experimentally by proteasome inhibition or forced expression of aggregating protein. Although calpains clearly can cleave large proteins into smaller fragments, which may be amenable to ubiquitination, we cannot exclude the possibility that calpains also have indirect effects on ubiquitin ligases or deubiquitinases that contribute to the observed effects.

It has been widely assumed that calpain activation has little relevance to normal cardiac physiology because calcium levels thought to be required for proteolytic activation are much greater than free cytosolic calcium levels observed in normal cells. However, association of calpains with cell membranes and binding of phospholipids greatly diminishes the calcium requirements for activation, suggesting that there may be significant biological effects under normal conditions. This is consistent with the current results, in which we detect calpain 1, but not...
calpain 2, activity in normal cardiac myocytes in culture and in situ, and in conditional transgenic hearts in vivo. A physiological function for calpains in skeletal muscle has previously been implicated by the occurrence of “calpainopathy,” or LGMD2A, in patients with loss of function mutations in the gene that encodes skeletal muscle-specific calpain 3.6,7 In this condition, diminished muscle calpain activity results in progressive weakness in the proximal limb-girdle muscles. Skeletal muscle biopsies of LGMD2A patients show variable skeletal myofiber size, myofiber atrophy, and patchy dystrophin staining,33,34 which resemble the histological findings in our cardiac calpain inhibition model, calpastatin overexpression. More recently, a role for calpain 3 in skeletal muscle ubiquitination and proteasomal degradation has also been suggested.5 The functional and pathological parallels between myocardial calpain 1 and skeletal muscle calpain 3 suggest that regulation of protein turnover via the ubiquitin/proteasome pathway may represent a general “physiological” function for calpains.

To define the essential function for myocardial calpains, we used a combination of loss-of-function analyses (adenoviral or transgenic expression of calpastatin) and gain-of-function analyses (overexpression of specific calpains). Although we believe that overexpression studies need to be interpreted with caution because the relevance of unregulated enzyme activity to naturally occurring pathophysiological processes is uncertain, the calpain transgenic models generated some interesting results. Notably, it appears that both calpains 1 and 2 are incompatible with life when expressed at high levels in the myocardium because we obtained no founder mice from constructs using the standard α-MHC promoter. When the less powerful tetracycline-suppressible bitransgenic α-MHC promoter system was used, modest overexpression of calpain 2 was tolerated, but levels of transgenic myocardial calpain 1 and 3 that were barely detectable at the protein level still caused massive myocardial necrosis and lethal heart failure. The calpain transgenic mouse models therefore demonstrate that calpain 1 has significant proteolytic activity for at least some of its known substrates in normal myocardium, which supports the notion that calpain inhibition by calpastatin produces the autophagic cardiomyopathy phenotype by interfering with normal physiological calpain-mediated proteolytic processes.

What of the calpain 1 transgenic phenotypes themselves? Our studies show that calpain 1 at high expression levels is sufficient to cause widespread myocytolysis and a striking inflammatory reaction, which in turn was sufficient to cause heart failure in mice. Because zymography shows that higher levels of overexpression of this protease result in higher endogenous calpain enzymatic activities, there may be an analogy between myocardial damage seen in the high expressing L3 calpain transgenic line and those conditions, such as ischemia/reperfusion, in which endogenous calpain 1 is activated by increased cytosolic calcium. Certainly, our data support the idea that calpain activation can contribute to myocardial injury. We further suggest that the physiological protein substrates of calpain 1, whose inhibition by calpastatin results in accumulation of aggregated protein and formation of autophagosomes described herein, are likely to differ from pathological substrates, such as the Bcl-2 family proteins9,30,46 and troponin I.10 Indeed, although we did not detect troponin I cleavage in calpain 1 transgenic hearts at baseline, it is readily detected after ischemic injury (A.D. and G.W.D., unpublished observations, 2006). Determining the relevance of differential processing of troponin I and other calpain substrates on the myocardial response to ischemic injury and on cardiac functional recovery to ischemic damage will require further investigation.

In conclusion, we have observed that myocardial calpain 1 is active under normal physiological conditions and may be positioned at the apex of an essential arm of the ubiquitin/proteasome degradation pathway that removes proteins whose abnormal accumulation causes cardiomyocyte degeneration and heart failure. Because the ubiquitin/proteasome system is, as its name implies, present in virtually every tissue, the observations that calpain 1 in the heart and calpain 3 in skeletal muscle are essential components of this degradative pathway suggest a broad role for calpains as critical regulators of cell protein turnover.

Figure 6. Schematic diagram depicting postulated role of calpain in proteasomal degradation of protein complexes (solid arrow) and shunting of misfolded proteins to autophagic pathway with aggresomal accumulation under conditions of calpain insufficiency.
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Disclosures

None.

References


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CARDIOMYOCYTE DEGENERATION WITH CALPAIN DEFICIENCY REVEALS A CRITICAL ROLE IN PROTEIN HOMEOSTASIS

SUPPLEMENTAL MATERIALS AND METHODS

Cell culture. Primary neonatal rat cardiomyocytes were prepared from hearts of 2- to 3-day-old Sprague-Dawley rat pups and grown in DMEM-M199 serum free medium for 24 h before infection. Adenoviral infections were performed at a multiplicity of infection of 100. For protein turnover studies, cultured cardiac myocytes were loaded with 2.5 μCi/ml 3H-tyrosine (NEN Perkin Elmer) overnight in tyrosine-free medium.

Immunoblot and Immunohistochemical Analyses. Polyclonal anti-calpastatin, anti-calpain 1, anti-PKCα, and anti-BclX$_{L/S}$ were from Santa Cruz Biotechnology, anti-calpain 2 and anti-calpain 3 (p94) were from Chemicon International, anti-Bid and anti-phospho-PKCα were from Cell Signaling Technology. Monoclonal anti-cardiac troponin I was from Spectral Diagnostics Inc., anti-dystrophin NCL-Dys1 mAb was from Novocastra, anti-desmin clone DE-U-10 was from Sigma, and anti-Bax was from Upstate Biotechnologies Inc. Protein ubiquitin content was assayed in the 125,000 x g pellet of cell or tissue extracts using mouse anti-ubiquitin from Zymed (Invitrogen) or monoclonal anti-polyubiquitin from Kamiya as described (1). Immunoblots were detected by peroxidase-conjugated enhanced chemiluminescence (Perkim Elmer Life Science). Equal membrane protein loading was verified by Ponceau S staining.

Cell viability was assessed with the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes). TUNEL was performed using the DeadEnd Fluorometric TUNEL System (Promega). For confocal studies, primary antibodies were visualized with anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes). Actin was stained with phalloidin/Alexa Fluor 568 (Molecular Probes). For ultrastructural studies, thin sections were counterstained with uranyl acetate and lead citrate and examined on a Zeiss 912 transmission electron microscope at an accelerating voltage of 100 kV.
Reference:

Supplementary Table 1. Conscious echocardiographics studies of Calpain 2 and Calpain 3 transgenic mice after neonatal induction.

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<th>Calpain 2 (neonatal induction)</th>
<th>Calpain 3 (neonatal induction)</th>
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<tr>
<td></td>
<td>NTG (n=7)</td>
<td>NTG (n=7)</td>
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<tr>
<td>LVEDD (mm)</td>
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<td>2.4±0.1</td>
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<td>LVESE (mm)</td>
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<td>1.4±0.1*</td>
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<tr>
<td>FS (%)</td>
<td>46±2</td>
<td>39±3*</td>
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<td>Wall thickness (mm)</td>
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<td>Heart rate (bpm)</td>
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Cardiac function in two weeks old Calpain 2 and Calpain 3 pups induced from birth. LVEDD=left ventricular end-diastolic dimension, LVESE=left ventricular end-systolic dimension, FS=fractional shortening. *=P<0.05 vs NTG.

Supplementary Table 2. Invasive hemodynamic data at baseline and in response to dobutamine.

<table>
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<tr>
<td>+dP/dt, base (mm Hg/s)</td>
<td>8,259±52</td>
</tr>
<tr>
<td>+dP/dt, Dob (mm Hg/s)</td>
<td>20,296±200</td>
</tr>
<tr>
<td>-dP/dt, base (mm Hg/s)</td>
<td>-8,568±332</td>
</tr>
<tr>
<td>-dP/dt, Dob (mm Hg/s)</td>
<td>-12,495±712</td>
</tr>
</tbody>
</table>

HR: Heart rate, bpm: beats per minute, Dob: value at peak dobutamine stimulation dose rate of 128 ng/g/min, LVP: maximum left ventricular pressure, dP/dt: peak rate of change of left ventricular pressure increase (+) or decline (-). *=P<0.05 vs NTG.
Supplementary Figure 1. Consequences of cardiac calpain 2 overexpression. (a) Zymography of calpain 2 activity in myocardial lysates from nontransgenic (NTG) and neonatal induced calpain 2 (Calp 2) mice. C = authentic calpain control. (b) Spectrophotometric assay of calpain activity in 5 mM calcium. Data are mean ± SEM (n=7-8), * indicates p=0.012 by t-test. (c) Heart weight corrected for body weight (HW/BW) in neonatal and adult mice with neonatal calpain 2 induction. (d) Immunoblot analysis showing absence of desmin or dystrophin (dys) cleavage in calpain 2 transgenic myocardium.
Supplementary Figure 2. Consequences of cardiac calpain 3 expression. (a) Kaplan-Meier survival curves with neonatal (clear) or adult (filled) induction. (b) Immunoblot analysis of calpain 3 expression. Overexpressed calpain 3 typically runs as a series of immunoreactive peptides in heart and skeletal muscle. (c) Heart weight corrected for body weight (HW/BW) in neonatal induced mice at 2 weeks of age and adult induced mice at 12 and 20 weeks of age. Data are mean ± SEM (n=6-10), ** indicates p<0.001 by t-test.
Supplemental Figure 3. Whole-heart electrical characteristics and atrial mechanical function in calpastatin hearts. (a) Six-lead EKGs show right axis deviation in CSTN. (b) Representative telemetry showing wide-complex tachycardia in CSTN, consistent with atrial tachycardia with aberrancy. (c) Representative Mitral inflow Doppler tracings demonstrating normal atrial activity (A wave) in nontransgenic mouse, but absent atrial contraction in tachycardic CSTN overexpressor. E wave is passive rapid ventricular filling.