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Lack of $\beta 3$ integrin signaling contributes to calpain-mediated myocardial cell loss in pressure-overloaded myocardium

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Abstract

Although cardiac hypertrophy initially ensues as a compensatory mechanism it often culminates in congestive heart failure. Based on our earlier studies that calpain and $\beta 3$ integrin play cell death and survival roles, respectively during pressure-overload (PO) hypertrophy, we investigated if the loss of $\beta 3$ integrin signaling is a potential mechanism for calpain-mediated cardiomyocyte death during PO. $\beta 3$ Integrin knockout ($\beta 3^{-/-}$) and wild type (WT) mice were used to induce either moderate or severe PO *in vivo* for short-term (72 h) and long-term (4 wk) by transverse aortic constriction (TAC). Whereas WT mice showed no changes during moderate PO at both time points, $\beta 3^{-/-}$ mice exhibited both enrichment of the μ -calpain isoform and programmed cell death of cardiomyocytes following 4 wk PO. However, with severe PO that caused increased mortality in both mice groups, cell death was observed in WT mice also. To study calpain's role, calpeptin, a specific inhibitor of calpain, was administered via osmotic mini pump at 2.5 mg/kg/day beginning three days prior to moderate TAC or sham surgery. Calpeptin administration blocked both calpain enrichment and myocardial cell death in the 4 wk PO $\beta 3^{-/-}$ mice. Since $\beta 3$ integrin contributes to cardioprotective signaling, these studies indicate that the loss of specific integrin function could be a key mechanism for calpain-mediated programmed cell death of cardiomyocytes in PO myocardium.

Keywords

cardiac hypertrophy; $\beta 3$ Integrin knockout mice; pressure-overload; programmed cell death

INTRODUCTION

Cardiovascular diseases such as hypertension, valvular defects, and myocardial infarction are often associated with the development of cardiac hypertrophy. Although hypertrophy begins as a compensatory phenomenon to an increase in hemodynamic load such as pressure overload, it often transitions into pathological remodeling and eventually into congestive heart failure (CHF) leading to increased morbidity/mortality. The specific mechanism behind the transition of compensation into CHF, however, is not well established. In this context, increased cardiomyocyte loss via programmed cell death as observed in human

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heart failure is thought to be one of the mechanisms for development of the maladaptive remodeling which culminates in heart failure (1, 2). Under these conditions, it is possible that the mechanism governing compensated growth and survival of cardiomyocytes is either altered or absent during sustained PO allowing programmed cell death to occur.

Recent studies in our laboratories show two divergent observations in PO myocardium: cardioprotective signaling via $\beta 3$ integrin-mediated cell survival mechanism (3) and programmed cell death of cardiomyocytes via calpain activation (4). In the case of $\beta 3$ integrin signaling, we utilized mice lacking $\beta 3$ integrin ($\beta 3^{-/-}$) to demonstrate that this integrin stimulation in PO myocardium is critical for NF κ B-mediated survival signaling, as its absence leads to enhanced programmed cell death of cardiomyocytes (3). Our parallel studies on calpain using a feline right ventricular pressure overload (RVPO) (4) and mouse myocardial infarction (MI) (5) models show that increased wall stress causes calpain activation leading to programmed cell death of cardiomyocytes.

Calpains are a family of Ca^{2+} -dependent cysteine proteases (6). The predominant isoforms include μ -calpain (calpain-I) and m-calpain (calpain-II). μ -calpain, a ubiquitously expressed isoform in all types of mammalian cells, is an 80-kDa protein that requires micromolar concentrations of Ca^{2+} for its activation. m-calpain is also 80-kDa in size, but requires millimolar concentration of Ca^{2+} , and its expression is higher in muscular tissue, kidney, brain, and liver. In the heart, numerous studies indicate that increased calpain activity might be a contributing factor to various pathological states, including hypertensive cardiomyopathy, myocardial infarction, and ischemia/reperfusion injury (7–14). Although multiple mechanisms may contribute to calpain activation, recent studies suggest that calpain activity is both positively and negatively regulated by specific integrins, subsequent to their engagement with cognate ECM proteins (15, 16). These studies indicate the importance of $\beta 3$ integrin-mediated survival signaling, absence of which leads to calpain-mediated cell death. Therefore, it is quite possible that $\beta 3$ integrin stimulation and the associated survival signaling during PO might play a positive role in preventing calpain activation, and the loss of this mechanism during chronic PO might lead to calpain activation causing cardiomyocyte loss. In the present study, this possibility was explored using $\beta 3^{-/-}$ mice that were subjected to TAC to induce left ventricular pressure overload (LVPO).

MATERIALS AND METHODS

Reagents

Calpeptin was obtained from Calbiochem (San Diego, CA). TUNEL staining kit was obtained from Chemicon (Temecula, CA). Antibodies against the following proteins were obtained from the indicated vendors: μ -calpain, m-calpain, active caspase-3 and α -actinin from Sigma Chemicals, MO, full-length caspase-3 antibody from Cell Signaling and fluorescent-labeled secondary antibodies from Molecular Probes, CA.

Animal Care

All animal studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy Press, Washington, DC, 1996) and were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

Knockout Mouse

The $\beta 3$ integrin knockout ($\beta 3^{-/-}$) mouse, originally created in the laboratory of Dr. Richard Hynes (MIT), was obtained from Jackson Laboratories. Genotyping for $\beta 3^{-/-}$ mice was conducted as per manufacturer's instructions. C57BL/6 mice were used as WT controls.

Murine TAC Model

This is an established mouse model of transverse aortic constriction (TAC) to produce LVPO. Microsurgical TAC or sham operation was described previously (3). Our previous work in WT mice with moderate PO by using 27-gauge needle during TAC surgery caused a 50% mortality rate during 4 wk TAC, and the surviving mice exhibited a compensated hypertrophy with preserved ventricular function (3). However, $\beta 3^{-/-}$ mice with this protocol exhibited higher mortality rate (80%) with decreased compensatory hypertrophy and function. Induction of severe PO using a 28 or 30-gauge needle caused severe mortality in $\beta 3^{-/-}$ mice (no surviving mice; $n=5$) and, to a lesser degree, in WT mice also (80%; $n=5$).

For moderate TAC, the transverse aorta was constricted by tying a suture around the vessel over a 27-gauge blunted needle causing occlusion of the aorta (3). The needle was withdrawn, resulting in a stenotic aortic lumen. For severe TAC, we used a 30-gauge needle. Three days and 4 wk after TAC surgery, animals were sacrificed by removal of the heart in deep anesthesia. For controls, sham operated mice without TAC was used.

Calpeptin Infusion

Adult (3 months old) WT and $\beta 3^{-/-}$ mice were anesthetized, and a small medial incision was made in the abdomen. The linea alba was carefully cut and the micro-osmotic pump (Alzet model 1004, Durect Corp, Cupertino, CA) was implanted intraperitoneally (*i.p.*). The pump contained calpeptin dissolved in DMSO that delivered calpeptin at 2.5 mg/kg/day or vehicle alone. Upon termination of the experiments, the mice were anesthetized with 5% isoflurane balanced with O_2 , and the heart was removed for histochemical and biochemical studies.

Confocal Imaging

Fresh LV tissue samples were embedded in tissue freezing medium (Optimal Cutting Temperature compound), and 12 μ m thick cryosections were prepared using a Leica cryomicrotome. Sections were used for immunostaining as described previously (4). Briefly, sections were blocked with 10% normal donkey serum for 1 h at room temperature and incubated overnight at 4°C with the following primary antibodies: rabbit anti- α -actinin (1:100), mouse anti- μ -calpain antibody (1:100) and rabbit anti-caspase-3 antibody (1:100). After washing sections thrice with PBS, Alexa-647-labeled anti-mouse IgG, Alexa-568-labeled anti-rabbit IgG were used as secondary antibodies and incubated for 2 h at room temperature. Nucleus was stained with TOPRO3 or DAPI (Molecular Probes, Carlsbad, CA). Apoptotic cell death was detected by TdT-mediated dUTP nick-end labeling (TUNEL) as per the manufactures protocol (Chemicon, Temecula, CA). The fluorescence staining was visualized using laser scanning confocal microscopy (Zeiss 510 Meta).

Western Blotting

Triton X-100 soluble proteins were extracted from LV samples and processed with SDS sample buffer as described previously (4). Proteins in SDS sample buffer were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked for 1 h using 1% BSA and 5% milk in TBST (10 mM Tris, 0.1 M NaCl, 0.1% Tween-20, pH 7.4). Blots were incubated with primary antibodies in TBST overnight at 4°C, washed five times,

each for five min with TBST and then incubated with horseradish peroxidase conjugated secondary antibody in TBST for 1 h at room temperature. Following five washes, each for five minutes with TBST, the proteins were detected by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

RESULTS

Calpain Activation in PO $\beta 3^{-/-}$ Mice

Both WT and $\beta 3^{-/-}$ mice were subjected to moderate PO via TAC for 72 h and 4 wk, and the activation of predominant calpain isoforms (μ and m) was studied by both immunohistochemical and Western blot analyses (Figure 1). Western blot analysis with both μ - and m -calpain antibodies showed the presence of these isoforms in the ventricular tissue samples, and there were no major changes observed in the levels of these two calpain isoforms (Figure 1A). Next, immunohistochemical analyses were performed similar to our recent studies (4, 5) where the LV tissue sections from WT and $\beta 3^{-/-}$ mice were stained with μ -calpain and α -actinin antibodies (Figure 1B). WT mice, either sham or moderate TAC for 72 h and 4 wk, showed only α -actinin staining of cardiomyocytes but not μ -calpain enrichment. In $\beta 3^{-/-}$ mice, both sham control and 72 h TAC also exhibited only α -actinin staining but not μ -calpain staining. However, $\beta 3^{-/-}$ mice undergoing long-term PO for 4 weeks readily showed μ -calpain enriched cardiomyocytes. Similar analysis for m -calpain did not show staining of this isoform either in PO LV tissue samples or sham controls of both mice types (data not shown), although m -calpain could be readily detected in these samples by Western blot analysis (Figure 1A). This suggested to us that only the μ -calpain isoform, and not the m -calpain isoform, becomes enriched/localized in the cell following PO. We therefore focused primarily on the μ -calpain isoform in our remaining experiments. We also tested the possibility of whether caspase-3 is activated during PO in $\beta 3^{-/-}$ mice. Western blot analysis using nonselective caspase-3 antibody detects caspase-3 in ventricular tissue samples (Figure 1C). Furthermore, in 4 wk PO tissue samples from both WT and $\beta 3^{-/-}$ mice showed a moderate increase in caspase-3 levels. However, immunostaining with active caspase-3 antibody showed no caspase-3 activity in the 4 wk PO ventricular tissue samples from WT or $\beta 3^{-/-}$ mice (Figure 1D). Similarly, caspase-3 staining was absent in samples from the 72 h TAC and sham operated control samples of both these mice types (data not shown). These studies indicate calpain, in particular μ -calpain, might be the endoprotease primarily activated in $\beta 3^{-/-}$ mice during long term (4 wk) moderate PO.

Programmed Cell Death of Cardiomyocytes in $\beta 3^{-/-}$ TAC Mice

Next, we analyzed whether calpain activation in 4 wk PO $\beta 3^{-/-}$ mice is accompanied by programmed cell death. For this, ventricular tissue samples from WT and $\beta 3^{-/-}$ mice that were subjected to either sham control, 72 h, or 4 wk PO were used for TUNEL staining as an indicator of DNA fragmentation and programmed cell death. Resulting data for sham controls and 72 h TAC mice for both WT and $\beta 3^{-/-}$ revealed no detectable levels of TUNEL positive cells (data not shown). Similarly, in the long-term PO WT mice for 4 wk, TUNEL was not present at a detectable level, and only nuclear staining was observed (Figure 2A). However, $\beta 3^{-/-}$ mice after 4 week of TAC showed programmed cell death as evidenced by an increased number of TUNEL positive nuclei (Figure 2A). These data observed in $\beta 3^{-/-}$ corroborate our earlier observations (3). To further demonstrate that TUNEL positive cells are cardiomyocytes enriched with μ -calpain, we immunostained for both calpain and α -actinin along with TUNEL (Figure 2B). These studies show that TUNEL positive cells in 4 wk PO $\beta 3^{-/-}$ mice costain with α -actinin and μ -calpain. Overall, the data suggest that programmed cell death as indicated by TUNEL positive cells in 4 wk PO $\beta 3^{-/-}$ mice might be mediated via activation of the μ -calpain isoform.

Calpain Inhibition Attenuates Programmed Cell Death in PO $\beta 3^{-/-}$ Mice

Next, we evaluated whether *in vivo* administration of calpeptin, a calpain inhibitor, could attenuate μ -calpain activation and prevent cardiomyocyte loss observed following a 4 wk PO in $\beta 3^{-/-}$ mice. Our recent study in PO felines demonstrates that calpeptin administration attenuated several PO-induced changes, including calpain enrichment and programmed cell death (4). Similar to our previous experiment, 4 wk PO in $\beta 3^{-/-}$ mice caused calpain activation accompanied by TUNEL positive cells (Figure 3). Administration of calpeptin at 0.5 mg/kg/day during the 4 wk period of PO blocked both calpain activation and TUNEL reactivity, indicating that the activated calpain isoform(s) in $\beta 3^{-/-}$ mice mediate programmed cell death. Importantly, this prevention of cell death of cardiomyocytes by calpeptin was accompanied by reduced mortality rate from 80% (n=5) in untreated group to 40% (n=5) in calpeptin treated group.

Calpain Activation and Programmed Cell Death in Severely PO WT Mice

Finally, to demonstrate that calpain activation and programmed cell death could be induced in WT mice, we performed severe PO using a 30-gauge needle. Experiments described in Figure 4 clearly indicate that severe PO causes both calpain activation and programmed cell death as early as in 72 h as evidenced by the presence of TUNEL and μ -calpain positive cells. Furthermore, this trend was present when the severe PO was continued for 4 wk (Figure 4). Therefore, under severe PO conditions, both calpain activation and programmed cell death occur in WT mice.

DISCUSSION

In the present study, we explored whether the absence of $\beta 3$ integrin stimulation during PO can induce calpain activation that leads to programmed cell death of cardiomyocytes. When compared to WT mice counterpart, $\beta 3^{-/-}$ mice shows: (i) enhanced calpain enrichment in cardiomyocytes even with moderate PO for 4 wk, (ii) some of the μ -calpain enriched cardiomyocytes stain positive for TUNEL, and (iii) administration of calpeptin that abolished μ -calpain enrichment also prevented programmed cell death. In support of these findings, our recent work both in PO feline myocardium (4) and in post-infarct overloaded viable murine myocardium (5) demonstrate that calpain activation is responsible for the accompanying cardiomyocyte death. Furthermore, our earlier work using $\beta 3^{-/-}$ mice demonstrates that $\beta 3$ integrin is critical both for the activation of NF κ B-mediated cell survival signaling and for accompanying expression of cellular inhibitors of apoptosis (cIAPs) in both PO myocardium and integrin-stimulated adult cardiomyocytes (3). Therefore, the loss of $\beta 3$ integrin-mediated survival signaling during PO is a potential mechanism for both calpain activation and associated programmed cell death of cardiomyocytes.

Loss of cardiomyocytes is considered an important feature during decompensatory hypertrophy (10, 17–20) where the activation of cellular proteases causes the cleavage of multiple cellular proteins, including sarcomeric proteins, leading to the loss of cardiomyocytes. Under these conditions, both cardiomyocyte loss and the impaired contractile function of remaining viable cardiomyocytes contribute to the transition of compensating heart into maladaptive remodeling resulting in CHF (17). In this context, numerous studies indicate that increased activities of both caspase-3 and calpain are involved in various pathological states including hypertensive cardiomyopathy, myocardial infarction, and ischemia/reperfusion injury (12). For example, calpain activation has been demonstrated in PO myocardium (4, 10, 14), Ca^{2+} -overloaded rat myocardium (21), spontaneously hypertensive rats (22), and isoproterenol induced hypertrophied hearts (13). Our present work indicates activation of calpain, in particular μ -calpain, in PO $\beta 3^{-/-}$ mice

that contributes to the observed programmed cell death of cardiocytes. This observation on μ -calpain is based on our findings in immunohistochemical studies where (i) 4 wk PO in $\beta 3^{-/-}$ mice myocardium showed enrichment of μ - but not m-calpain isoform, although both these isoforms were detected by Western blot analysis (Figure 1) and (ii) treatment with calpeptin, a specific inhibitor of calpain, blocked both μ -calpain activation and programmed cell death of cardiomyocytes in PO $\beta 3^{-/-}$ mice. Therefore, $\beta 3$ integrin-mediated signaling plays a role in suppressing μ -calpain activation in hypertrophying myocardium. Although caspase-3 activation is critical for apoptotic cell death, our immunohistochemical analyses reveal that the increased level of TUNEL positive cardiomyocytes in PO $\beta 3^{-/-}$ mice was not accompanied by this protease activation (Figure 1D). Furthermore, caspase-3 is known to undergo cleavage where a decreased level of full-length caspase-3 is indicative of its activation (23). However, our Western blot analysis in 4 wk PO tissue samples using non-selective caspase-3 antibody readily detected caspase-3, although it did not show a decreased level of caspase-3 but instead showed a moderate increase (Figure 1C). In support of these observations, our previous work in PO feline myocardium (4) demonstrates programmed cell death of cardiomyocytes accompanied by calpain activation, and these changes could be blocked by treatment with a calpain inhibitor (calpeptin) but not with a caspase inhibitor (Z-VD-fmk). Together, these studies reveal that μ -calpain may function as a major isoform activated during chronic PO in $\beta 3^{-/-}$ mice that mediates programmed cell death of cardiomyocytes.

In the present study, the increased TUNEL reactivity in $\beta 3^{-/-}$ mice was similar to our earlier work in these mice (3) where the level of TUNEL positive cells following a 4 wk PO was estimated as 2%. Although TUNEL positive nuclei primarily represent cell death via apoptotic mechanism, both cells undergoing necrotic death and viable cells undergoing DNA repair were also found to exhibit TUNEL reactivity (24–26). Therefore, the cell death that we observe in the current study could be partly due to apoptosis, necrosis or both which are collectively referred here as programmed cell death. Furthermore, although TUNEL assay reveals cells with damaged DNA, which could be higher than the actual number of dying cells, previous studies in transgenic animals have shown that cardiocyte loss as low as 0.02% is sufficient to cause lethal dilated cardiomyopathy (1). In addition to such deleterious effects, even the viable cardiomyocytes that have not completed the cell death process might also contribute to the loss of ventricular function. Therefore, 2% TUNEL reactivity in PO $\beta 3^{-/-}$ mice myocardium can be anticipated to have a severe pathological consequence. In the present study, administration of calpeptin that blocked μ -calpain enrichment in 4 wk PO $\beta 3^{-/-}$ mice both prevented cell death of cardiomyocytes as evidenced by the absence of TUNEL staining and reduced mortality rate from 80% in untreated group to 40% in calpeptin treated group. Cardiomyocyte loss in PO myocardium is not unique to $\beta 3^{-/-}$ mice, since induction of severe PO in WT mice resulted in both calpain activation and programmed cell death of cardiomyocytes (Figure 4). As expected, severe PO had higher mortality rate in $\beta 3^{-/-}$ mice (no surviving mice following 4 wk PO; n=5). Together, these data indicate that PO triggers a calpain-mediated cell death process especially when $\beta 3$ integrin-mediated signaling is compromised, and this could be a potential mechanism for the observed cardiocyte loss in PO myocardium.

Of the several possible mechanisms explaining how the loss of $\beta 3$ integrin signaling might contribute to the programmed cell death of hypertrophying cardiomyocytes, one potential mechanism is based on previous studies in multiple cell types (15, 16, 27, 28) where changes in the extracellular matrix (ECM) environment was found to cause the loss of $\alpha v \beta 3$ integrin signaling that could result in the activation of calpain and programmed cell death. Furthermore, the loss of $\alpha v \beta 3$ integrin activation is known to reduce NF κ B-mediated cell survival signaling (3, 29, 30), and therefore, it is possible this condition favors calpain-mediated programmed cell death. Analogous to this, during both hemodynamic overload and

post infarction overload to the heart, increased ventricular wall stress triggers ECM degradation, especially of collagen, by the activated matrix metalloproteinases (31, 32). Thus, the generation of collagen fragments in PO myocardium may lead to calpain activation and programmed cell death via a similar mechanism. Alternatively, PO-induced cardiac hypertrophy is known to cause endoplasmic reticulum (ER) stress (10), resulting in both the release of calcium and activation of calpain (10, 33–36), and this sequence of events is known to promote programmed cell death. Whether the loss of $\beta 3$ integrin signaling during PO generates excessive ER stress leading to the observed calpain activation remains to be established.

Overall, the present study demonstrates that the loss of $\beta 3$ integrin signaling during PO could be a potential mechanism for calpain activation, leading to programmed cell death of cardiomyocytes. Therefore, calpain and its upstream activators involving integrins could be possible targets to prevent programmed cell death in patients with hypertensive heart diseases.

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Fig.1 A,B

Fig. 1C,D

Figure 1.

μ -calpain activation in moderately PO myocardium. Age-matched WT (C57BL/6) mice and $\beta 3^{-/-}$ mice were subjected to sham surgery or moderate TAC with 27-gauge needle for the indicated times ($n \geq 4$ /group) and analyzed by Western blot and immunofluorescence. (A) LV tissue samples were extracted with Triton X-100 buffer and the soluble protein samples were used for Western blot analyses using anti- μ -calpain, -m-calpain and -GAPDH antibodies. (B) Fresh frozen LV tissue sections from control and 72 h and 4 wk moderately PO mice were stained with anti- μ -calpain antibody (blue) and anti- α -actinin antibody (red). (C) LV tissue samples from control and 72 h and 4 wk moderately PO mice were extracted with Triton X-100 buffer and the soluble protein samples were used for Western blot analyses using anti-caspase-3 (full-length) antibody. (D) Fresh frozen LV tissue sections from 4 wk PO mice groups were stained with anti-active caspase-3 antibody (blue) and anti- α -actinin antibody (red). Scale bar=10 μ M.

Figure 2. μ -calpain enrichment and programmed cell death of cardiomyocytes in moderately PO myocardium. **(A)** LV tissue samples from 4 wk moderate TAC mice (WT and $\beta 3^{-/-}$) were processed for TUNEL (green) and nuclear (DAPI, red) staining as described in Materials and Methods ($n \geq 4$ /group). **(B)** LV tissue samples from 4 wk TAC mice (WT and $\beta 3^{-/-}$) were processed for TUNEL (green) and μ -calpain (blue) staining as described in Materials and Methods ($n \geq 4$ /group). Scale bar=10 μ M.

Figure 3.

Effects of calpeptin treatment on μ -calpain enrichment and programmed cell death of cardiomyocytes in moderately PO myocardium. Age-matched WT mice and $\beta 3^{-/-}$ mice were administered calpeptin at 0.5 mg/kg/day via Alzet micro-osmotic pump three days prior to TAC, and the drug was continued during the 4 wk moderate TAC period ($n \geq 4$ /group). LV tissue samples from WT (data not shown) and $\beta 3^{-/-}$ mice were processed for TUNEL (green), α -actinin (red) and μ -calpain (blue) staining as described in Materials and Methods ($n \geq 4$ /group). Scale bar=10 μ M.

Figure 4.

μ -calpain enrichment and programmed cell death of cardiomyocytes in severely PO WT mice myocardium. WT mice were severely PO using a 30-gauge needle for 72 h or 4 wk. Control mice were sham operated and sacrificed after 4 wk. (*Top Panel*) LV tissue samples from control and TAC mice for 72 h and 4 wk were processed for TUNEL (green) and α -actinin staining. (*Bottom Panel*) LV tissue samples from control and 72 h and 4 wk severe TAC were processed for μ -calpain (blue) and α -actinin staining as described in Materials and Methods ($n \geq 3$ /group). Scale bar=10 μ M.