Spatio-temporal regulation of calpain activity after experimental myocardial infarction in vivo

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ABSTRACT

Background: Calpains are calcium activated cysteine proteases that play a pivotal role in the pathophysiology of cardiac remodeling.

Methods: Here, we performed left anterior descending coronary artery ligation in rats as a model for ischemic systolic heart failure and examined the time- and region-specific regulation of calpain-1 and calpain-2 in the left ventricular myocardium.

Results: Following anterior wall myocardial infarction, calpain activity was significantly increased restricted to the ischemic anterior area at days 1, 5 and 14. No changes in calpain activity at neither time point were detected in the borderzone and remote posterior area of the left ventricle. Of note, calpain activity in the infarcted anterior myocardium was regulated differentially in the acute vs. subacute and chronic phase. In the acute phase, calpain translocation to the plasma membrane and attenuation of the expression of its endogenous inhibitor, calpastatin, were identified as the driving forces. In the subacute and chronic phase, calpain activity was regulated at the level of protein expression that was shown to be essentially independent of transcriptional activity.

Conclusions: We conclude that myocardial infarction leads to a distinct calpain regulation pattern in the left ventricular myocardium that is region specific and time dependent. Considering the results from our previous studies, a spatio-temporal interaction between calpains and calcium dependent natriuretic peptide production in the infarcted myocardium is possible.

General significance: Our results shed more light in the differential regulation of calpain activity in the myocardium and might aid in the development of targeted post-infarct and/or heart failure therapeutics.

Abbreviations: AGTR1, angiotensin II receptor type 1; InsP3, inositol 1,4,5-trisphosphate; InsP4, inositol 1,4,5-trisphosphate receptor; LAD, left anterior descending; LVEDD, left ventricular enddiastolic diameter; LVEF, left ventricular ejection fraction; LVESD, left ventricular endsystolic diameter; NF-κB, nuclear factor kappa B; NT pro-ANP, N-terminal pro atrial natriuretic peptide; SBP, spectrin breakdown products.

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1. Introduction

Calpains are calcium activated cysteine proteases expressed in the cytoplasm. They play a pivotal role in heart failure being involved in the pathophysiology of cardiac remodeling processes such as hypertrophy, inflammation and fibrosis [1]. They have been examined in numerous experimental settings including loss-of-function and gain-of-function studies [2–4]. Strategies to target calpain as a therapeutic approach in heart failure have been sought in the past years.

Under physiological conditions, calpains contribute to maintain normal cell and tissue function through protein processing and degradation. Their activity is controlled by calpastatin, which is a specific endogenous calpain inhibitor. Since calpains are calcium activated proteases, imbalances in calcium homeostasis, which e.g. occur during hypoxia, ischemia and post-ischemic reperfusion, can result in disturbed calpain activity in the myocardium [5–7].

The role of calpains in myocardial infarction as the leading cause for heart failure has been widely studied, but conflicting results have been reported. It has been shown that calpain inhibition leads to decreased infarction size, improved remodeling and survival [3,8–10]. However, other studies showed that inhibition of calpain’s activity can lead to degenerative conditions [11–13]. Even though targeting calpain for ischemic heart failure treatment seems promising, the mechanism of calpain related signaling and function turned out to be quite complex and therefore needs further investigation.

The aim of this study was to characterize calpain expression and activity in the myocardium after experimental myocardial infarction in vivo in a time- and region-specific manner to help better understand calpain regulation and function within the heart.

2. Material and methods

2.1. Experimental myocardial infarction

Ligation and sham surgery of the left anterior descending (LAD) coronary artery in male Wistar rats (Janvier Labs and Charles River) was performed as previously described [14–16]. Animals were allocated to one of the two groups. Surgical procedures were conducted under anesthesia with 8% chloral hydrate and endotracheal intubation. Carprofen 5 mg/kg body weight was applied for analgesia. Animals were re-anesthetized and sacrificed at the end of the observation period according to study group allocation after 1, 5 or 14 days. Exclusion criteria were lack of epicardial cyanosis, lack of hypokinesia or akinesia in echocardiography and lack of ST-segment elevation in ECG immediately after LAD ligation. All animal procedures were performed in agreement with the "Guide for the Care and Use of Laboratory Animals" and were approved by the local ethics committee (#24-9168.11-1/2013-49).

Fig. 1. Animal model characteristics. A) ECG. ST-elevation (white arrow, right panel) immediately after ligation of the LAD confirms transmural myocardial infarction (representative recordings). Left panel: ECG prior to LAD ligation. Right panel: ECG immediately after ligation of the LAD. B) Echocardiography. Left ventricular ejection fraction (LVEF) at days 1, 5 and 14 after myocardial infarction (day 1: control 88.3 ± 1.6% vs. ligation 65.6 ± 2.3%; day 5: control 85.1 ± 2.4% vs. ligation 52.9 ± 3.5%; day 14: control 87.7 ± 2.0% vs. ligation 47.4 ± 4.2%; n = 10). C) Plasma natriuretic peptides. NT pro-ANP elevation at days 1, 5, 14 after myocardial infarction (day 1: control 1.04 ± 0.21 nmol/L vs. ligation 1.71 ± 0.24 nmol/L; day 5: control 1.67 ± 0.16 nmol/L vs. ligation 2.38 ± 0.35 nmol/L; day 14: control 1.67 ± 0.29 vs. ligation 3.02 ± 0.39; n = 9–10). D) Histopathology, representative short axis slices. Visualization of tissue myocardial remodeling after myocardial infarction at days 1, 5 and 14. Stainings are with hematoxylin and eosin (left column) and picro-sirius red (right column). The ischemic anterior wall (infarct zone – IZ) shows necrosis already at day 5 (*). After 14 days, the anterior wall is thinned and primarily constituted by connective tissue (infarct zone - IZ). Fibrosis is also obvious in the borderzone (BZ). The non-infarcted remote zone (RZ) responds with compensatory hypertrophy. *p < 0.05; **p < 0.01; ***p < 0.001. Lig – ligation group; sham – control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
2.2. Histology

Whole hearts were taken and fixed in 4% formaldehyde, embedded in paraffin and sectioned at 1 mm intervals with a thickness of 4 μm each (rotation microtome from Leica Biosystems). Hematoxylin/eosin and picro-sirius red stainings were performed using standard procedures as described [17].

2.3. Echocardiography

M-mode measurements to determine left ventricular enddiastolic and endsystolic diameters (LVEDD, LVESD) were performed using the S12 transducer (Sonos 5500, Philips) in anesthetized animals. Left ventricular ejection fraction (LVEF) was calculated. To increase the accuracy of measurements, each diameter was determined threefold, and the mean value calculated thereof.

2.4. Protein measurement and immunoblotting

Protein measurements were performed according to the method of Bradford [18] after homogenization of tissue samples. To obtain the membrane fraction for calpain translocation analysis, we used the modified method by Strasser et al. [19]. In brief, frozen rat heart tissue was homogenized in lysis buffer followed by a low speed centrifugation step to remove unbroken cells and nuclei (pellet). The supernatant was collected and used for high speed centrifugation, resulting in a pellet being the membrane fraction and the supernatant as the cytosolic fraction. The high grade of membrane fraction purity obtained by this method has been demonstrated before [20]. Immunoblotting was performed as previously described [21,22]. The following antibodies and dilutions were used: anti-αII-spectrin 1:1000 (Enzo), anti-calpain-1 1:1000 (Cell Signaling Technology), anti-calpain-2 1:1000 (Cell Signaling Technology), anti-calpastatin 1:1000 (Cell Signaling Technology). Either anti-β-actin 1:1000 (Santa Cruz) or Ponceau S staining (Sigma Aldrich) were used as loading controls. Quantitative analyses were carried out by densitometry using Quantity One and Fiji ImageJ. Samples were determined in single measurements.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Plasma levels of N-terminal pro atrial natriuretic peptide (NT pro-ANP) were determined by using a commercially available kit according to the manufacturer’s protocol (BIOMEDICA BI-20892 pro ANP [1–98]). For statistical analysis, each sample was determined twofold, and the mean calculated thereof.

2.6. Real time PCR

Real time PCR was used to quantify mRNA expression of selected genes. Total RNA was isolated from heart tissue using the Universal RNA Purification Kit (Roboklon) according to the manufacturer’s protocol. An on-column DNase treatment was conducted to eliminate genomic DNA. The Revert AID™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used to perform reverse transcription. Real-time PCR for the quantification of the cDNA template was performed using the Maxima SYBR Green Kit (Thermo Fisher Scientific) as described previously [23]. Primers were obtained from Eurofins Genomics GmbH (Supplementary Table 1). POLr2A und TBP were used as housekeeping genes, and the Vandesompele method was applied for quantification [24]. Samples were determined in duplicates.

2.7. Statistical analysis

Microsoft Office Excel 2016 und GraphPad Instat® Version 3.05. were used for statistical analyses. The data are presented as mean ± standard error of the mean (SEM). Between-group differences were assessed using the independent t-test or one-way ANOVA, where appropriate. Post hoc analyses were assessed via HSD Tukey Kramer.

Fig. 2. Region- and time-specific regulation of calpain activity after infarction. A) Calpain activity, assessed by the accumulation of 145/150 kDa αII-spectrin breakdown product (αII-SBDP) is increased restricted to the infarcted anterior myocardium. B) Representative Western blot showing increased expression of αII-SBDP in the infarcted anterior wall; β-actin served as loading control. C) The amount of αII-spectin protein expression in the ischemic, borderzone and posterior regions at days 1, 5 and 14. D) Representative Western blot showing αII-spectrin protein expression in the ischemic wall; β-actin served as loading control. E) Ratio of αII-SBDP/αII-spectrin. *p < 0.05; **p < 0.01; ***p < 0.001. Lig – ligation group; sham – control group.
test. A p-value < 0.05 was considered statistically significant, * indicates the level of significance with *p < 0.05; **p < 0.01; ***p < 0.001.

3. Results

3.1. Animal model characterization

We performed ligation of the LAD coronary artery to induce ischemic myocardial infarction with acute systolic heart failure. Successful induction of myocardial infarction and development of heart failure were assessed by ECG, echocardiography, plasma natriuretic peptides and histopathology (Fig. 1).

3.2. Spatio-temporal regulation of calpain activity

To characterize post-ischemic calpain activity of myocardial tissue, we chose three time points for coverage of the acute (1 day), subacute (5 days) and chronic (14 days) stage.

Calpains are proteases that hydrolyze plenty of substrates [25]. Hydrolyzation of αII-spectrin leads to 145/150 kDa spectrin breakdown products (SBDP), the amount of which reflects calpain activity and is enzyme-specific [26]. Our results show that αII-SBDP are significantly increased up to 4-fold in the ischemic anterior zone starting at day 1 after myocardial infarction (day 1: control 0.43 ± 0.05 vs. ligation 2.24 ± 0.36 [n = 10], p < 0.001; day 5: control 0.46 ± 0.07 vs. ligation 2.18 ± 0.44 [n = 10], p < 0.01; day 14: control 0.82 ± 0.09 vs. ligation 2.95 ± 0.29 [n = 10], p < 0.001). The borderzone and posterior region did not show significantly altered calpain activity at any time point (Fig. 2A and B, values in Supplementary Table 2).

To rule out that increased amounts of αII-SBDP in the ischemic anterior wall was based on an increase of αII-spectrin total amount, we determined the expression of αII-spectrin (Fig. 2 C and D). At day 1 after infarct, there was no difference in αII-spectrin protein expression, indicating that the increased formation of αII-SBDP was indeed due to increased calpain activity. At days 5 and 14, expression of αII-spectrin was increased, however, clearly less pronounced compared to the increase of αII-SBDP, illustrated in Fig. 2E (ratio of αII-SBDP/αII-spectrin).

Furthermore, seeking for a confirmation of our results, we assessed cadherin protein expression levels of myocardial tissue. Cadherin proteolysis, in contrast to the accumulation of αII-SBDP, is an unspecific indicator of calpain activity. Results show that infarct zone cadherin protein levels are reduced at all three time points and again this effect is restricted to the infarcted anterior wall myocardium (Fig. 3A and B, values in Supplementary Table 2).
3.3. Assessment of calpain activity regulation

3.3.1. Calpain protein expression

To assess calpain activity regulation, we first studied calpain protein expression in all three zones at days 1, 5 and 14 after myocardial infarction. In concordance to the detected increase of calpain activity that is restricted to the anterior infarcted region, we found significant increases of calpain-1 protein expression restricted to the anterior ischemic wall only and again sparing the acute stage (day 1: control 0.76 ± 0.07 vs. ligation 0.57 ± 0.10 [n = 10], p = 0.117; day 5: control 0.91 ± 0.05 vs. ligation 0.72 ± 0.06 [n = 10], p = 0.011; day 14: control 1.00 ± 0.03 vs. ligation 0.82 ± 0.05 [n = 10], p = 0.001).

Fig. 4. Region- and time-specific regulation of calpain-1 protein expression in ischemic myocardium. A) Increase of calpain-1 protein expression is restricted to the infarct zone, but notably was not significantly altered before day 5. B) Representative Western blot demonstrating the increase of calpain-1 protein expression in the anterior wall in the subacute und chronic phase after myocardial infarction. C) Protein expression of calpain-2 is significantly increased in the anterior ischemic wall only and again sparing the acute stage (day 1). D) Representative Western blot demonstrating calpain-2 protein expression in the anterior wall after myocardial infarction at the three different time points studied. *p < 0.05; **p < 0.01; ***p < 0.001. Lig – ligation group; sham – control group.

3.3.2. Calpain regulation at the transcriptional level

A) Expression of calpain-1 mRNA was not altered in any of the three regions of the myocardium at any of the three time points after myocardial infarction. B) Expression of calpain-2 mRNA was not altered in any of the three regions of the myocardium at any of the three time points after myocardial infarction. Lig – ligation group; sham – control group.

Fig. 5. Calpain regulation at the transcriptional level. A) Expression of calpain-1 mRNA was not altered in any of the three regions of the myocardium at any of the three time points after myocardial infarction. B) Expression of calpain-2 mRNA was not altered in any of the three regions of the myocardium at any of the three time points after myocardial infarction. Lig – ligation group; sham – control group.
0.07 vs. ligation 1.79 ± 0.17 [n = 10], p < 0.001; day 14: control 1.13 ± 0.09 vs. ligation 2.28 ± 0.18 [n = 10], p < 0.001). No significant changes of calpain-1 protein expression were seen in the borderzone and remote posterior region at any time point (Fig. 4A and B, values in Supplementary Table 2).

Even more than calpain-1, calpain-2 protein expression was increased in the anterior infarcted myocardium and again sparing the acute phase, i.e. day 1 (day 1: control 0.77 ± 0.09 vs. ligation 0.52 ± 0.11 [n = 10], p = 0.1; day 5: control 0.88 ± 0.11 vs. ligation 2.69 ± 0.35 [n = 10], p < 0.01; day 14: control 1.32 ± 0.11 vs. ligation 4.17 ± 0.82 [n = 10], p < 0.01) (Fig. 4C and D). Again, essentially no significant changes in protein expressions were observed in the borderzone and remote posterior region at any time point (values in Supplementary Table 2).

3.3.2. Calpain mRNA levels
Looking at the transcriptional level, neither calpain-1 nor calpain-2 mRNA expressions were significantly altered in any of the three regions of the myocardium at any time point (Fig. 5, values in Supplementary Table 2).

3.3.3. Calpastatin protein expression
Calpastatins are specific endogenous inhibitors of calpains. To further investigate the regulation of calpain activity, we examined calpastatin protein expression at days 1, 5 and 14 after myocardial infarction in the infarct zone, borderzone and remote zone (Fig. 6A and B, values in Supplementary Table 2). In parallel to our results showing significantly altered calpain activity in the anterior infarct zone only, significant changes of calpastatin protein expression were detected only in the anterior region, notably starting immediately at day 1. At day 1 after myocardial infarction, calpastatin protein expression was significantly decreased, whereas at days 5 and 14 we saw a significant increase (day 1: control 0.93 ± 0.13 vs. ligation 0.37 ± 0.08 [n = 10], p < 0.01; day 5: control 0.90 ± 0.10 vs. ligation 1.53 ± 0.12 [n = 10], p < 0.001;
day 14: control 1.22 ± 0.11 vs. ligation 1.99 ± 0.14 (n = 10), p < 0.001; values normalized to ß-actin).

3.3.4. Calpastatin mRNA levels
Calpastatin mRNA levels were not changed in any of the regions at neither of the three time points (Fig. 6C, values in Supplementary Table 2).

3.3.5. Plasma membrane translocation of calpains
To further examine the increase of calpain activity at day 1, when calpain protein expression was still unchanged, we investigated the membrane translocation of calpains. Membrane translocation of calpains has been previously described to facilitate calpain activation by different mechanisms and therefore also was considered in our study. We observed significant translocation of calpain-1 to the plasma membrane in the anterior infarct zone, exclusively in the acute phase at day 1 (Fig. 7A, values in Supplementary Table 2). For calpain-2, a similar tendency was detected, although statistical significance was not reached in this set of experiments (Fig. 7B, values in Supplementary Table 2).

4. Discussion
In the present article, we studied calpain activity and its regulation in vivo in a rodent ischemic heart failure animal model. We demonstrate a spatial dependence of calpain activity in the myocardium that can be explained by spatio-temporally distinct regulatory mechanisms comprising calpain protein expression, calpain translocation to the plasma membrane and the presence and differential regulation of calpain’s endogenous inhibitor, calpastatin.

Validity and suitability of our animal model was proven by several findings: I) ST-segment elevation on ECG as a sign of transmural myocardial infarction as well as immediate pale-off of the infarcted region by visual assessment, II) decreased LVEF on echocardiography, III) increased plasma NT pro-ANP levels, and IV) histopathology demonstrating characteristic post-infarct tissue remodeling at three different time points, i.e. acute phase at day 1, subacute phase at day 5 and chronic phase at day 14.

We describe a significant rise of calpain activity that, notably, was restricted to the infarcted anterior myocardium and starting immediately at day 1 (acute phase) after experimental myocardial infarction. The increase of calpain activity in the acute phase was not attributable to changes of calpain protein expression, because calpain-1 and calpain-2 protein levels were not substantially changed at day 1. Therefore, regulation of calpain activity in the acute phase following myocardial infarction seems to be controlled differently. We provide evidence that this is achieved by 2 different but complementary mechanisms. 1) We demonstrate that at day 1, calpains translocate to the plasma membrane. There, various factors can facilitate calpain activation, such as conformational changes, membrane phospholipids and locally concentrated calcium [27,28]. 2) Protein expression of calpastatin, calpain’s endogenous inhibitor, is significantly suppressed in the anterior infarcted region in the acute stage; up-regulation of calpastatin protein expression starting at day 5 probably occurs in the sense of a negative feedback loop protecting the myocardium from overwhelming calpain activity that would otherwise result from the strong increase of calpain protein expression levels in the infarcted myocardium starting at day 5.

In the subacute and chronic phase, the increase of calpain activity is
Calpain activation depends on calcium (Ca$^{2+}$). In the myocardium, one important mechanism of calcium liberation form internal stores is via angiotensin II receptor type 1 (AGTR1) activation that stimulates inositol 1,4,5-trisphosphate receptors (InsP$_3$R). InsP$_3$R open probability is regulated by chromogranin B (CGB). Several downstream targets of calpain signaling are proposed, including nuclear factor kappa B (NF-$\kappa$B). B) Infarcted myocardium – acute phase. The increase of calpain’s catalytic activity within the infarcted myocardium in the acute phase is mediated by calpain translocation to the plasma membrane, where multiple factors facilitate calpain activation. Complementary, a reduction of overall calpastatin protein expression is observed. C) Infarcted myocardium – subacute and chronic phase. The increase of calpain’s catalytic activity within the infarcted myocardium in the subacute and chronic phase, instead, is mediated by up-regulation of total calpain protein expression that exceeds a concomitant (but smaller) relative increase of calpastatin (cs) protein expression. AGTR1 - angiotensin II receptor type 1. ANP - atrial natriuretic peptide. Ca$^{2+}$ - calcium. CGB - chromogranin B. CS - calpastatin. InsP$_3$ - inositol 1,4,5-trisphosphate. InsP$_3$R - inositol 1,4,5-trisphosphate receptor. NF-$\kappa$B - nuclear factor kappa B. The “pink star” represents calpain’s catalytic activity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

5. Limitations

(1) This study is descriptive in its nature and aimed to describe the spatio-temporal activation pattern of calpain-1 and calpain-2 in the ventricular myocardium following experimental myocardial infarction. We describe temporally distinct mechanisms of calpain activation in the anterior wall myocardium in the acute stage vs. the subacute and chronic stages. To clarify the mechanisms behind different protein expression levels is an important aspect that still needs to be addressed but was beyond the scope of the current study.

(2) The results of this study are obtained in cardiac tissue of the left ventricular myocardium, consisting of cardiomyocytes, fibroblasts, and endothelial cells. In the infarcted area, immune cells and myofibroblasts also contribute to the tissue. Therefore, our results cannot be attributed to one specific cell type (e.g. cardiomyocytes), but are representative for the tissue analyzed.

6. Conclusion

We demonstrate in a rodent myocardial infarction animal model that calpain activity and protein expression in the left ventricular myocardium are distinctly regulated in a spatio-temporal pattern, exclusively covering the infarcted area with potential implications on the production of natriuretic peptides. Our results shed more light in the differential regulation of calpain activity in the myocardium and might aid in the development of tailored heart failure therapeutics in the future.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101162.

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