Ph.D. THESIS

ANALYSIS OF μ-CALPAIN-MEDIATED *IN VITRO* PROEOLYSIS OF HUMAN MYOCARDIAL PROTEINS

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Introduction

Calpains and their possible role in the myocardium

Calpains are Ca^{2+}-activated intracellular neutral proteases. They are present in most of the eukaryotic cells. The role of calpains in the myocardium is partly understood. They have been implicated in the regulation of individual tissue protein levels, in the development of ischemic/reperfusion injury (myocardial stunning), in protein metabolism, thus in the regulation of growth, differentiation and atrophy of the myocardial tissue. Two tissue-specific calpain types are expressed in the myocardium. μ-calpain (or calpain-1), requires Ca^{2+} concentration in the μM range for *in vitro* activation. Remarkably higher Ca^{2+} (mM) is necessary to activate the other calpain type, m-calpain. The precise mechanism of intracellular calpain activation presently not fully understood, since the Ca^{2+} concentration required for their proteolytic activity is higher than 50-300 nM found in living cells. It is believed, that the Ca^{2+} concentration required by the autolysed μ-calpain is in the physiological range. The Ca^{2+} concentration required for activation and function by m-calpain *in vitro* is most probably incompatible with cell viability.

At low intracellular Ca^{2+} concentration calpains are associated with myofibrillar and cytoskeletal structures (mainly sarcomer Z-bands) in the cytosol in inactive form. The enzyme is translocated into the cell membrane upon increase in Ca^{2+}, and becomes activated following intramolecular proteolysis. The function of calpains is regulated via other mechanisms in addition to Ca^{2+} and autoproteolysis, like endogous calpain inhibitor (calpastatin), which prevents spontaneous calpain activation, phospholipids, that regulate the Ca^{2+}-sensitivity of the enzyme and endogenous calpain activators. Unfortunately, the physiological role and regulation of calpains remain enigmatic in the myocardium. However, limited proteolysis is typical feature of calpain proteolytic activity, which results in stabile, large protein fragments. This feature indicates posttranslational modification instead of protein degradation. Therefore, μ-calpain is believed to be the regulator of the cell protein metabolism (house-keeping enzyme), rather than degradative enzyme.

Familial hypertrophic cardiomyopathy and calpains

Familial hypertrophic cardiomyopathy (fHCM) is characterized by ventricular hypertrophy, myofibrillar disarray, fibrosis and is often linked to a high incidence of sudden cardiac death. More than 100 point-mutations in 9 genes, all coding thin and thick filament sarcomeric proteins, have been linked to the disease. Numerous mutations of the cardiac troponin I (cTnI)-encoding gene also evoke fHCM. cTnI is the inhibitory component of the troponin complex (cTnI : troponin T (cTnT) : troponin C (cTnC) = 1 : 1 : 1). Different regions of this polypeptide, made up of 210 amino acid residues, interact with actin, cTnC and tropomyosin during the cardiac cycle Ca^{2+}-dependent manner. This function of cTnI is tightly related with the structure of the molecule. Minor changes in the structure of cTnI may result in change in myocardial contractiliy. Motivated by the wealth of information pointing to the central position of cTnI in the regulation of the contractile process, intensive research has been conducted in an effort to understand the consequences of cTnI gene mutations on the myocardial mechanics. In fact, most of the studies initiated on the basis of this functional concept revealed that the Ca^{2+}-sensitivity of force production and of myosin ATPase activity were increased in certain preparations involving the fHCM-related cTnI mutants. It is noteworthy, however, that the mutations affect structurally and functionally distinct regions of cTnI that do not uniformly modulate the forces of contraction, the cTnC-binding and the myosin ATPase activity. Conversely, hypertrophies with similar cardiac morphologies result from point mutations of remote regions of the cTnI molecule. For example, the R145G mutation, which alters the structure of the central inhibitory domain, and the K206Q mutation in the C-terminal region both induce the “ventricular” type of hypertrophy. Surprisingly, another mutation in the C-terminal region (G203S) is associated with the “apical” type of hypertrophy. Thus, the mutations in different cTnI regions and their functional consequences on the myofibrillar mechanics do not provide a full explanation of the morphology and clinical characteristics of fHCM.

It is known, that the calpains are involved in muscle growth and differentiation. Therefore, it is possible that calpains play a role in the regulation of muscle protein turnover (degradation and assembly), which theoretically can lead to the shift in muscle protein-mass and myocardial hypertrophy under pathological conditions. Experiments on various animal models indicated that μ-calpain is involved in the proteolytic cleavage of wilde type cTnI in

reversible postischemic myocardial dysfunction, i.e. myocardial stunning. Based on these data it has been hypothesized that fHCM-associated mutations in cTnI amino acid sequence leads to an altered susceptibility of the different mutant cTnI molecules (R145G, G203S, K206Q) to μ-calpain-induced proteolysis. The decrease in μ-calpain-mediated degradation of cTnI results in accumulation of the mutant proteins and is responsible for the changes in contractile function and/or morphological characteristics of fHCM.

Protein kinase-A-mediated cTnI phosphorylation and its consequences

The fine-tuning of troponin function is mediated by phosphorylation/dephosphorylation, which is tightly related with the conformation of the troponin molecules. Intracellular phosphorylation of cTnI is catalysed by protein kinase-A (PKA). cTnI has two PKA phosphorylation sites with functional importance in the position of Ser-22 and Ser-23. Therefore, the molecule may exist in three forms with different phosphorylation states in the myocardium: dephosphorylated, monophosphorylated and bisphosphorylated. The distribution of these forms is dependent on the actual activity level of the β-adrenergic system. The PKA-mediated phosphorylation of cTnI induce a decrease in Ca^{2+}-sensitivity of the contractile system, however, both phosphorylation sites need to be phosphorylated for this effect. The phosphorylation of cTnI enhances myocardial relaxation, since Ca^{2+}-affinity of cTnC is decreased, which results in faster Ca^{2+} dissociation. Furthermore, PKA induce the phosphorylation of phospholamban, the sarcoplasmic reticulum Ca^{2+} pump regulator protein, which leads to a faster sequestration of the cytoplasmic Ca^{2+}. This enhanced lusitropic ability is particularly beneficial during β-adrenergic stimulation, via compensating the advers effects of the increased heart rate.

Experience shows, that hearts with dilated cardiomyopathy (New York Heart Association (NYHA) class III-IV.) and low left ventricular function are more susceptible to myocardial stunning compared to the healthy hearts. The difference in PKA phosphorylation level of the myocardial proteins gives possible explanation for this observation. PKA phosphorylation level of certain proteins i.e. cTnI and phospholamban is decreased in heart failure due to the down-regulation of the β-adrenergic system. According to the literature, cTnI phosphorylation level is approximately 84 % in the healthy and only 56 % in the failing myocardium. The decrease in cTnI phosphorylation level observed in heart failure, induce an

increase in Ca^{2+}-sensitivity of the myofibrillar system. The change in phosphorylation level leads to an altered charge and conformation of the cTnI molecule. The Ca^{2+}-bound, opened conformation is stabilized upon cTnI dephosphorylation, which is shifted to the Ca^{2+}-dissociated, closed conformation upon phosphorylation. It was suggested that phosphorylation-dependent conformational change of cTnI can modify the accessibility of the molecules for proteolitic enzymes, i.e. μ-calpain. The molecules become more susceptible to calpains upon dephosphorylation. Experiments using PKA or β-adrenergic stimulation to induce cTnI phosphorylation provide indirect evidence for this observation, in these experiments a decrease in μ-calpain susceptibility of cTnI was observed upon phosphorylation.

Proposed mechanism of reversible ischemic/reperfusion injury of the myocardium – calpain theory

The reversible postischemic myocardial dysfunction known as myocardial stunning is a frequently occurring clinical syndrome. Changes in myocardial cell metabolism and Ca^{2+}-homeostasis are believed to be responsible for the contractile dysfunction. The low energy supply compared to demand during ischemia leads to the disturbance of the most energy-demanding functions of the myocardium, i.e. Ca^{2+}-homeostasis. Therefore, we have to face elevated intracellular Ca^{2+} concentration. There is a fast recovery in extracellular pH and oxygen upon reperfusion, however reperfusion paradoxically induces a transitory intracellular Ca^{2+} overload. Furthermore, reoxygenation of the ischemic myocardium induces an intensive burst of oxygen radicals, which plays a role in destroying the membrane network separating the cell compartments with high Ca^{2+} concentration, further increasing the intracellular Ca^{2+} overload. Based on the facts mentioned above, Ca^{2+} overload during late ischemia and early reperfusion is a key player in myocardial ischemic/reperfusion injury.

There is strong experimental evidence that fundamental alterations occur in the contractile apparatus as a result of the concerted action of free radicals and intracellular Ca^{2+} overload. It is believed that Ca^{2+} overload is the trigger of the Ca^{2+}-dependent μ-calpain activation and consecutive protein degradation. In particular, the degradation of cTnI has been suggested as a crucial molecular defect of the contractile apparatus, which makes a major contribution to the development of postischemic myocardial stunning. This hypothesis

was supported by the results of model experiments on rat hearts, by the detection of cTnI degradation in ventricular tissue samples from individuals undergoing coronary bypass surgery, and by findings on a transgenic mouse model that expressed the truncated cTnI molecule ($cTnI_{1-193}$). This latter one established a strong link between cTnI degradation and a compromised cardiac function. μ-calpain has been implicated in the proteolysis of cTnI in rat hearts. μ-calpain decreased the Ca^{2+}-activated force and its Ca^{2+} sensitivity both in permeabilized rat cardiac trabeculae and myocyte-sized preparations; hence, these changes reproduced those reported earlier for postischemic myofibrillar preparations from the same species. However, in the myocyte-sized preparation these mechanical alterations occurred in the apparent absence of cTnI degradation. Additionally, myocardial stunning in large animal models was devoid of cTnI degradation. It is noteworthy that, in different animal models, the degradation of cTnT, myosin light chain-1 and various other myofibrillar proteins, e.g. titin, α-fodrin (also called non-erythroid α-spectrin) and α-actinin has been suggested to be involved in the postischemic myofibrillar dysfunction, these proteins being cleaved by μ-calpain.

A number of research laboratories have set out to pinpoint the key molecular events leading to the contractile dysfunction, using various animal models. Unfortunately, extrapolation from animal studies to the human heart is problematic, due to inherent conflicts between the models applied and the human pathology. Hence, the proteins undergoing limited proteolysis by μ-calpain in human myocardial stunning still need to be identified.

Aims

In vitro experiments were performed to answer the following questions:

1. Is there an effect of fHCM-associated point-mutations (R145G, G203S és K206Q) on calpain-sensitivity of recombinant cTnI?
2. Is there a difference in μ-calpain-susceptibility between the isolated and the complexed mutant cTnI molecules?
3. Is there an effect of PKA-mediated bisphosphorylation on μ-calpain-susceptibility of the mutant cTnI molecules?
4. Does the relation between cTnI mutations and μ-calpain-susceptibility give explanation for the morphological and functional changes found in fHCM?
5. Is there a difference in the cTnI phosphorylation level between donor hearts and hearts with dilated cardiomyopathy (NYHA III-IV)? Does it affect their μ-calpain-sensitivity?
6. Are there regulatory and structural proteins in the human myocardium sensitive to μ-calpain in addition to cTnI? Is there a difference in their μ-calpain-sensitivity?
7. How comparable are the results found in animal models of myocardial stunning and *in vitro* μ-calpain-mediated digestions of myobibrillar proteins?
8. What are the possible proteolytical changes during uncontrolled μ-calpain activation in the human myocardium?

Materials and Methods

Expression and purification of human cardiac troponin molecules

Human recombinant cardiac troponin molecules: cTnI (wild type (WT) and mutants (R145G, G203S and K206Q)), cardiac troponin T (cTnT) and cardiac troponin C (cTnC) were provided by Kornelia Jaquet et al. (Ruhr-Universität Bochum), obtained as described previously by Deng et al. and Reiffert et al.

Human heterotrimeric troponin complexes were reconstituted by mixing cTnT, cTnC and either WT or mutant cTnI (R145G, G203S and K206Q) at a molar ratio of 1:1:1 in a buffer containing 6 M urea. This was followed by dialysis against high salt buffer with decreasing concentrations of urea, and the salt content of the protein was then reduced stepwise. Reconstitution of the cTn complex was checked by analytical gel filtration (Pharmacia Sephadex G-75).

WT and mutant cTnI molecules were bisphosphorylated at Ser-22 and Ser-23, using the catalytic subunit of protein kinase-A as described previously by Deng et al. This procedure resulted in comparable phosphorylation degrees for all the different cTnI molecules. The phosphorylation states of cTnI were routinely controlled by isoelectric focusing. Only those proteins were used that contained no dephosphorylated forms and contained less or equal than 10% monophosphorylated and more or equal than 90% bisphosphorylated forms.

Tissue samples

Human left ventricular myocardial tissue samples were obtained from five different individuals. Three samples were taken from donor hearts that were not used for heart transplantation because of technical limitations. Two additional cardiac biopsies were excised during ventricular volume-reducing operations on patients (NYHA III-IV.) with dilated cardiomyopathy. All biopsies were frozen in liquid nitrogen and stored at –80 °C. All experimental procedures were approved by the Ethical Committee of University of Debrecen Medical and Health Science Centre, complying with the Helsinki Declaration of the Word Medical Association.

In vitro proteolysis with μ-calpain

Purified human nonphosphorylated and PKA-bisphosphorylated cTnI molecules (3 μg of the isolated WT and R145G, G203S and K206Q mutants; 10 μg of the cTn complexes with the above cTnI molecules) were incubated in Ca^{2+}-containing imidazole-buffer at 30 ^{o}C in volumes of 100 μl containing, pH 7.5. Proteolysis was initiated by adding of 0.05, 0.1, 0.5, 1, 2.5 U μ-calpain (Calbiochem) to the reaction mixtures. After incubation for 0.5, 30, 60 or 120 min, 23 μl aliquots were collected and boiled in SDS sample buffer for 5 min. Incubations in the presence of 0.3 mM Calpain Inhibitor 1 (Calbiochem) or in the absence of Ca^{2+} served as controls.

Small pieces of left ventricular tissue (about 5 mm^3) were pulverized in a chilled mortar and homogenized in ice-cold Ca^{2+}-free imidazole-buffer, pH 7.5. All reaction mixtures contained Phosphatase Inhibitor Cocktail I (1 μl/500 μg protein; Sigma). Protein concentration was determined according to the Bradford method. The crude homogenates (600 μg total protein) were subsequently incubated at 30 ^{o}C in total volumes of 200 μl. Proteolysis was initiated by the addition of 1 U or 5 U μ-calpain (Calbiochem) and 3 mM $CaCl_2$ to the reaction mixtures. After incubation for 0.5, 15, 30, 60 or 120 min, aliquots were collected and immediately boiled in SDS sample buffer for 3 min. Mixtures incubated in the presence of 0.3 mM Calpain Inhibitor I (Calbiochem) or in the absence of Ca^{2+} served as controls. Incubations in the absence of μ-calpain were used to test the endogenous Ca^{2+}-dependent proteolytic activity.

One-dimensional gelelectrophoresis

To ascertain the μ-calpain-induced proteolytic changes in the crude ventricular tissue homogenates, one-dimensional continuous-gradient sucrose-SDS-polyacrylamide gelelectrophoresis (Protean II xi, Bio-Rad) was performed in a non-stacking gel system (concentration range: 6-18%). For further analysis of the selected proteins, equal amounts of protein homogenates were separated on single-concentration SDS-polyacrylamide gels as described by Laemmli.

Silver staining

The protein pattern of the homogenates was visualized with silver staining as described by Giulian et al. Silver Standard (Bio-Rad) was used for protein molecular weight estimation.

Western immunoblot analysis and evaluation of cTnI proteolysis

Western immunoblot was performed to identify the selected proteins. Briefly, gels were transferred to nitrocellulose membranes with a wet transfer apparatus (Bio-Rad). Membranes were incubated with 5% non-fat dry milk, followed by incubation with primary monoclonal antibodies (anti-titin, anti-α-fodrin, anti-α-actinin, anti-desmin, anti-cTnT, anti-cTnI) and with peroxidase-conjugated secondary antibody. Enhanced chemiluminescence was used for detection. Prestained Standard (Bio-Rad) was used for protein molecular weight estimation.

Determination of phosphorylation level of cTnI

Ca^{2+}-free tissue homogenates were prepared on ice in the presence of Phosphatase Inhibitor Cocktail I (1 μl/500 μg protein) and boiled in equal volumes of SDS sample buffer to maintain the *in vivo* level of phosphorylation of the proteins. Optical density versus protein concentration calibration curves were produced to estimate the protein amounts giving the unsaturated Western immunoblot signal intensities for the total and non-phosphorylated cTnI-specific monoclonal antibodies. Equal amounts of the protein homogenates of each sample were then separated on SDS polyacrylamide gels and Western immunoblotting was performed with the two cTnI-specific antibodies (total and non-phosphorylated cTnI-specific antibodies). The measured optical densities (for the two antibodies) were utilized to express the quantities of the phosphorylated and non-phosphorylated cTnI percentages of the total cTnI.

Statistics

Band intensities of silver-stained gels and Western immunoblots were quantified from unsaturated recordings by densitometry, using NIH ImageJ 1.30v software.

Protein degradation of recombinant molecules was expressed as the relative decrease in intensity of the 31 kDa cTnI specific protein band. The independent digestions were repeated 4-10 times.

Qualitative differences in the intensity of μ-calpain proteolysis in myocardial homogenates were estimated on the basis of the activity- and time-dependences of the decay of the full-length intact proteins and of the accumulation of the degradation products.

Proteins, which underwent faster degradation at a given μ-calpain activity were considered more susceptible to μ-calpain proteolysis than those which degraded more slowly. The low temperature (4 ^{o}C) and the absence of Ca^{2+} suppressed the endogenous tissue proteases before the test incubations, and heat-denaturation terminated the enzymatic reactions after them. The results of protein degradations in the presence of added μ-calpain were not corrected for endogenous Ca^{2+}-dependent proteolysis. All experiments on all different tissue samples were repeated at least 3 times.

Calculated values of protein phosphorylation are expressed as means ± S.E.M. Differences were tested by means of Student's *t*-test at a level of significance $P<0.05$.

Results

μ-calpain-sensitivity of recombinant WT and 3 fHCM-related mutant cTnI (G203S, K206Q, R145G) was compared to reveal if fHCM development is related to altered μ-calpain-sensitivity.

Degradation of WT cTnI as a function of μ-calpain-activity and time

Equal amounts of WT cTnI was incubalted in the presence of increasing μ-calpain activity. The disappearance of the protein band probed with anti-TnI antibody in immunoblots at 31 kDa (native cTnI) indicated cTnI specific protein degradation. Additionally, the applied monoclonal antibody identified a cTnI degradation product at 26 kDa. At low μ-calpain activities (0.05 U and 0.1 U), relatively long incubation (120 min) was required to provoke appreciable cTnI degradation. High μ-calpain activities (0.5 U, 1 U and 2.5 U), however, accelerated cTnI proteolysis. In the presence of high μ-calpain activities, the cTnI specific bands had disappeared completely after 120 min of incubation, indicating that native cTnI and its visible breakdown product were degraded into fragments smaller than 26 kDa. cTnI truncation could be prevented either by omitting Ca^{2+} from the reaction mixtures ("control") or by the inclusion of 0.3 mM calpain-specific inhibitor (Calpain Inhibitor 1). Calpain Inhibitor 1 prevented the proteolysis (in the presence of 1 U μ-calpain and Ca^{2+}) of all the cTnI molecules tested. Hence, the detected degradations in cTnI must be specific to μ-calpain

and there were no contaminating proteases present in our reaction mixtures. 0.25 U (low activity, inducing moderate WT cTnI degradation) and 1 U (high activity, inducing extensive WT cTnI degradation) μ-calpain were used for further experiments.

Proteolytic effect of 1U μ-calpain on WT and mutant cTnI

Different cTnI molecules (WT cTnI and fHCM-related cTnI mutants) were incubated in the presence of 1 U μ-calpain and Western immunoblots were performed following test incubation for 0.5, 30, 60 or 120 min. The rapid disappearance of the 31 kDa cTnI specific band of the dephosphorylated samples (isolated or complexed WT and R145G, G203S and K206Q mutant cTnI molecules) demonstrates that fHCM-related mutations do not prevent μ-calpain-induced proteolysis. The faint bands at 26 kDa (seen occasionally for all the different dephosphorylated cTnI molecules) suggested low levels of the cTnI degradation product. The appearance of this degradation product was, however, not associated with a specific cTnI sequence. The cTnI specific bands of the bisphosphorylated forms at 31 kDa were partially preserved during the entire course of μ-calpain incubation under identical experimental conditions. Thus, phosphorylation at Ser-22 and Ser-23 opposed the proteolytic effect of 1 U μ-calpain for the WT and mutant cTnI molecules. Protein phosphorylation provided qualitatively similar protection against μ-calpain-induced proteolysis for isolated cTnI molecules and for the cTnI molecules in the troponin complex.

Proteolytic effect of 0.25 U μ-calpain on WT and mutant cTnI

To assess whether there are differences in the susceptibility of the various cTnI molecules (isolated WT and mutant cTnI molecules alone or in the troponin complex) for μ-calpain, the test incubations were performed in the presence of a reduced μ-calpain activity (0.25 U). This did not eliminate cTnI immunoreactivity following 30 min of μ-calpain proteolysis, and hence allowed an assessment of the amount of undegraded cTnI proteins at this time point. The susceptibilities of the various cTnI forms to degradation were estimated on Western immunoblots on the basis of the changes in the densitometric intensities of the 31 kDa cTnI band between 0.5 and 30 min of incubation with μ-calpain. The results illustrate that 0.25 U μ-calpain decreased the amounts of isolated WT, R145G, G203S and K206Q mutant cTnI

molecules to 22±9%, 27±10%, 25±7% and 45±6%, respectively. In contrast, the amounts of cTnI in the complexed WT, R145G, G203S and K206Q mutant cTnI molecules were significantly ($P<0.05$) higher (87±13%, 89±8%, 75±5% and 82±10%, respectively) after 30 min of μ-calpain proteolysis. However, no significant differences in the relative protein amounts were observed when the different cTnI molecules were compared with each other in the two groups ($P>0.05$). Thus, the cTnI gene mutations did not modify the susceptibility to μ-calpain proteolysis.

Differences in PKA phosphorylation level of cTnI in donor and DCM hearts and its effect on μ-calpain-mediated degradation

It is known that phosphorylation level of cTnI is significantly lower in failing hearts compared to donors. It has been demonstrated that cTnI molecules with low levels of protein kinase-A-mediated phosphorylation are significantly more susceptible than the fully phosphorylated cTnI molecules to μ-calpain proteolysis. To establish whether the cTnI phosphorylation level of the DCM hearts is decreased, phosphorylation level of cTnI was determined in all the different samples (2 DCM and 3 donor hearts) by using an indirect screening method. Western immunoblotting, with an antibody not sensitive to cTnI phosphorylation (clone 16A11), revealed that the total cTnI contents in the homogenates from the 5 different hearts were similar. However, when a primary antibody specific for non-phosphorylated cTnI (Ser22/Ser23) was used (clone 22B11), stronger signal intensities were observed for the samples from individuals with dilated cardiomyopathy than for those from the donors. The calculated levels of phosphorylation of the cTnI molecules in the samples of the patients with dilated cardiomyopathy and in those of the donors were $37 \pm 7\%$ and $62.5 \pm 2\%$ ($P<0.05$), respectively. Despite this difference in the cTnI phosphorylation levels, however, we found no appreciable differences between the cTnI degradation intensities of the donor and the dilated hearts.

Overall proteolytic effect of 5 U μ-calpain on crude myocardial homogenates

The proteolytic effects of μ-calpain are reflected by the results of silver-stained gradient SDS-polyacrylamide gelelectrophoresis. When the protein profiles of a Ca^{2+}-free control tissue homogenate and of a sample incubated for 120 min in the presence of 5 U μ-calpain

were compared, marked decreases in protein band intensities were noted at 12 different positions. Decreases in protein band intensities (even the complete disappearance of bands) or the appearance of additional bands upon μ-calpain treatment suggested the occurrence of proteolytic degradation. Many such changes occurred in a wide range of molecular mass. Some of them appeared at molecular masses similar to those of myofibrillar proteins implicated previously in myocardial postischemic injury. However, there were no appreciable differences between the protein profiles of the tissue homogenates before and after incubation for 120 min in the absence of Ca^{2+}, or in the presence of 0.3 U Calpain Inhibitor I and 5 U μ-calpain.

Changes in myofibrillar protein pattern induced by low and high μ-calpain activity

The previous experimental findings led us to test whether the observed proteolytic changes involve the μ-calpain-mediated proteolysis of a set of myofibrillar proteins: titin, α-fodrin, α-actinin, desmin, cTnT and cTnI. Degradation of these proteins was further investigated by appropriate Western immunoblotting or SDS-polyacrylamide gel analyses in order to compare their μ-calpain susceptibilities. Results of test incubations with μ-calpain activities of 1 U or 5 U were evaluated after 0.5, 15, 30, 60 or 120 min. The intensification of proteolysis at the higher μ-calpain activity revealed that all of the selected proteins were degraded by μ-calpain. Additionally, careful comparison of the proteolytic alterations at the two different μ-calpain activities allowed the recognition of differences in their μ-calpain susceptibilities.

Titin degradation was displayed on silver-stained SDS gels, and the locations of the two intact titin isoforms (N2B and N2BA with molecular masses of 3000 kDa and 3300 kDa, respectively) were verified by Western immunoblot analysis. μ-calpain degraded both isoforms of this giant structural protein and produced 7 degradation products between ≈3000 kDa and ≈1700 kDa. Both isoforms were completely proteolyzed by 1 U μ-calpain within the first 15 min of incubation. The higher μ-calpain activity (5 U) accelerated titin degradation and resulted in several degradation products. Overall, titin proved very susceptible to μ-calpain proteolysis. The structural protein α-fodrin was also very sensitive to μ-calpain. Western immunoblot assays revealed that the intact α-fodrin molecule (284 kDa)

was quickly cleaved at the lower μ-calpain activity (1 U). Moreover, its first degradation product (≈164 kDa appeared at the beginning of the test incubation and, similarly to titin, the full-length molecule had disappeared after 15 min of incubation. In the presence of 5 U μ-calpain, another proteolytic fragment was observed at ≈130 kDa. The Z band structural protein desmin (64 kDa) too was largely damaged by μ-calpain. However, the disappearance of the intact molecule was slower than that of titin or α-fodrin. Although, several desmin degradation products (≈54, 52, 45, 44, 41, 39 and 37 kDa) appeared rapidly after the beginning of the incubation, at the lower (1 U) μ-calpain activity intact protein could still be detected even after 120 min of incubation. The higher μ-calpain activity (5 U), however, led to a dramatic decrease in the level of the intact molecule and quickly eliminated the intermediate-sized degradation products. cTnT (44 kDa), the tropomyosin-binding member of the regulatory troponin complex, similarly exhibited an intermediate susceptibility to μ-calpain proteolysis. The full-length molecule and its single degradation product (≈33 kDa) were present throughout at the lower μ-calpain activity (1 U). In the presence of the higher μ-calpain activity (5 U), the degradation product of cTnT predominated and the full-length cTnT molecule quickly disappeared. Under the same conditions cTnI (31 kDa), the inhibitory unit of the troponin complex, underwent a clearly visible, but less intense degradation than those of all the above proteins. Although several cTnI-immunospecific products (≈28, 27, 26, 23, 19, 18, 16 and 13 kDa) were present during the incubations with μ-calpain, even the higher enzyme activity (5 U) failed to induce total degradation of this molecule by the end of the 120-min incubation period. α-actinin (115 kDa), the actin-binding structural protein of the Z bands, was the least sensitive of these proteins to μ-calpain. However, the clear appearance of double bands (≈116 kDa and ≈112 kDa, respectively) in the presence of 5 U μ-calpain suggested its susceptibility to μ-calpain-mediated proteolysis. Comparison of the results obtained on the 5 different cardiac samples failed to reveal significant interindividual differences in the proteolytic profiles of the investigated proteins. Interestingly, we found cTnI with relatively low μ-calpain susceptibility compared to titin, α-fodrin, desmin and cTnT.

Endogenous Ca^{2+}-dependent proteolytic activity in the human myocardium

We set out to compare the result of Ca^{2+}-dependent proteolytic activity of the human cardiac homogenates with the exogenous μ-calpain-provoked proteolysis. This was achieved by incubating the tissue homogenates in the presence of Ca^{2+} and in the absence of μ-calpain for 120 min. Western immunoblots revealed that, even in the absence of exogenously added μ-calpain, titin, α-fodrin, desmin, cTnI and cTnT underwent partial proteolysis: the larger titin isoform disappeared, one of the two α-fodrin degradation fragments and numerous immunoreactive products of desmin and cTnI became detectable, and the reduction in signal intensity of the intact cTnT was also indicative of proteolysis. However, α-actinin remained intact under the same conditions. The extent of this autodegradation generally accounted for less than 5% of the intact molecules (except in the case of titin). Comparison of the molecular mass distributions of the above autoproteolytic fragments with those generated by μ-calpain revealed several matching fragments of α-fodrin, desmin and cTnI. This suggests that at least part of the endogenous Ca^{2+}-dependent proteolytic activity could be mediated by calpains.

Discussion

The potential role of μ-calpain in the development of fHCM and myocardial stunning was investigated in the present study.

The role of μ-calpain in fHCM

The accumulation of muscle mass is a consequence of a disturbed balance between protein synthesis and protein degradation. Calpains have been implicated in the turnover of muscle proteins. Hence, we decided to establish whether there is a relationship between μ-calpain-mediated proteolysis and specific mutations of cTnI that cosegregate with fHCM. The effects of conformational changes due to troponin complex formation and protein kinase-A-mediated phosphorylation on the proteolysis intensity were also tested.

Most of our knowledge on cTnI breakdown originates from experiments on ischemic/reperfused hearts of rodents. It was earlier recognized that cTnI degradation occurs in several steps in the postischemic myocardium of the rat. Following the initial loss of a 17 amino acid long peptide from the C-terminus, subsequent proteolytic steps truncate the N-terminus by 62, and then by an additional 10 residues. The correspondence between the molecular masses of the cTnI specific bands demonstrated in this study and others (ischemic/reperfused rat hearts, and cardiac biopsies of individuals undergoing coronary artery bypass surgery) suggests that our antibody (clone C5) identifies the first cTnI degradation product ($cTnI_{1\text{-}193}$). Further, this finding extends earlier observations on rat hearts to human WT and mutant cTnI molecules and supports the theory of μ-calpain-induced cTnI degradation in the pathogenesis of postischemic stunning. The complete loss of cTnI immunoreactivity following intensive μ-calpain digestion indicates that μ-calpain may be responsible for at least two subsequent truncation steps, and that the epitope of our antibody is located within the N-terminal fragment of cTnI.

It is currently not known how the mutations in functionally different regions of cTnI (i.e. at the C-terminus (G203S and K206Q) and in the central inhibitory domain (R145G)) induce fHCM. The G203S mutation evokes the apical type of hypertrophy, characterized by a spade-shaped left ventricle. In contrast, K206Q and R145G were detected in the more frequent ventricular type of hypertrophy. Expression of the truncated cTnI ($cTnI_{1\text{-}193}$)

molecule in transgenic mice provoked cardiac enlargement. However, histological and biochemical markers of the remodeled ventricles in this transgenic line were not consistent with those found in another transgenic line expressing the mutation equivalent to the R145G mutation of fHCM (R146G in the mouse sequence) or ventricular hypertrophy in humans. Thus, $cTnI_{1\text{-}193}$ is probably not involved in fHCM. Alternatively, a decrease in the breakdown of mutant cTnI molecules may theoretically contribute to the increase in muscle mass during fHCM. The results of this study, however, show that none of the mutations prevented μ-calpain digestion. In line with previous observations, we found that μ-calpain-mediated proteolysis was generally less intensive when cTnI was assembled into the troponin complex. However, we could not find significant differences in the proteolytic intensities when WT and mutant cTnI molecules were compared with each other either in isolation or in the troponin complex. Hence, differences in the μ-calpain-sensitivities of the fHCM-related cTnI mutations do not provide a plausible explanation for the development of either type of fHCM.

The effect of PKA-mediated recombinant cTnI phosphorylation on μ-calpain-mediated degradation

Intracellular PKA phosphorylates two Ser residues (Ser-22 and Ser-23) close to the N-terminus of cTnI upon cardiac β_1-receptor stimulation. The cTnI conformation that develops as a consequence decreases the apparent Ca^{2+}-sensitivity of force production, and thereby contributes to an enhanced relaxation during sympathetic activation. Conversely, most of the cTnI mutations found in fHCM increase the Ca^{2+}-sensitivity of force production, and hence affect the relaxation adversely. The results of a recent *in vitro* motility study demonstrated that the phosphorylation-dependent regulatory properties of the R145G mutant are largely compromised. Although, the combined effects of cTnI mutations and cTnI phosphorylation on the myofibrillar mechanics are not fully understood, there is a growing consensus that an impairment of ventricular relaxation may initiate the hypertrophic transformation of the myocardium during fHCM. Our results on recombinant cTnI molecules are in concert with previous studies on cardiac preparations of the rat, which suggested that phosphorylation of cTnI confers protection against μ-calpain-mediated proteolysis. Here we provide evidence that cTnI mutations in the central inhibitory domain (R145G) and in the C-terminal region (G203S and K206Q) do not modulate bisphosphorylation-mediated protection against μ-

calpain proteolysis. Additionally, we demonstrate that bisphosphorylated WT and mutant cTnI molecules are also protected against μ-calpain proteolysis in the troponin complex.

In conclusion, the R145G, G203S and K206Q mutations of cTnI did not affect μ-calpain-induced cTnI degradation, nor its PKA-mediated prevention of μ-calpain proteolysis. Thus, protein phosphorylation may effectively influence the intensity of cTnI degradation in fHCM during ischemic attacks. Moreover, major differences in the μ-calpain-dependent degradation of various cTnI mutants might not be probable unless there are differences in the phosphorylation levels of the cTnI mutants in the myocardium or in the functions of intracellular calpains during fHCM.

The effect of PKA-mediated human myocardial cTnI phosphorylation on μ-calpain-mediated degradation

Further studies revealed that the resistance of bisphosphorylated cTnI against ischemia-induced or μ-calpain-mediated proteolyses in the rat and also in the human myocardium is not present on the mono- and nonphosphorylated forms. The phosphorylation levels of the cTnI molecules in our human samples were comparable with those found in previous investigations; accordingly, phosphorylation of cTnI cannot explain its relatively low μ-calpain susceptibility. Moreover, in line with these experimental findings, the cTnI in the samples that were obtained from remodeled ventricles was less phosphorylated. However, no significant difference in μ-calpain susceptibility was observed between tissue samples with different levels of cTnI phosphorylation. Since our method did not discriminate between the mono- and bisphosphorylated cTnI molecules, a plausible explanation for this apparent discrepancy is that the amounts of bisphosphorylated cTnI were similar in the donor and the dilated hearts, while the amount of monophosphorylated cTnI was significantly higher in the donor than in the dilated hearts.

The role of μ-calpain in myocardial stunning

Transient μ-calpain activation and subsequent limited myofibrillar proteolysis have been proposed as the explanation of postischemic myocardial stunning. The results of our present investigation relate to a number of human myofibrillar proteins as potential targets of μ-

calpain. The degradation of these proteins by the uncontrolled intracellular activation of μ-calpain may possibly disturb the structure and the regulation of the contractile machinery in the human heart *in vivo*.

A wealth of information on cTnI degradation in ischemic/reperfused myocardia indicates an altered contractile regulation. cTnI undergoes truncation in the postischemic myocardium of rat heart in at least three steps. The first clipping removes 17 amino acids from the C-terminus, while the subsequent ones truncate the N-terminus by 62, and then by an additional 10 residues. The molecular masses of the cTnI specific degradation bands demonstrated in this study overlap with those found in other preparations, such as ischemic/reperfused rat hearts and human left ventricular tissues. However, our antibody (clone 16A11) identified more than three cTnI degradation products within the same range of molecular masses. Hence, μ-calpain may have cleaved human cTnI molecules at more sites than the number deduced from postischemic rat hearts. This may possibly explain the large number of cTnI modification products in epicardial biopsy samples obtained from bypass patients. Alternatively, the cleaved cTnI fragments could form covalent complexes with other myocardial proteins, giving a rise to a number of cTnI immunoreactive bands.

In a previous study, μ-calpain-induced desmin degradation was paralleled by a partial loss of the sarcomere pattern and by decreased Ca^{2+}-activated force generation in permeabilized rat cardiomyocytes. Here, we demonstrate two additional structural myofibrillar proteins (i.e. titin and α-fodrin) with higher susceptibilities for μ-calpain than that of desmin in the human myocardium. These data and the similarities in the degradation products extend earlier observations on various animal preparations to the human myocardium and support the involvement of their proteolysis in myocardial stunning in man. Titin is a giant structural protein that spans between the Z bands and the M lines and is the main intracellular contributor to the passive tension. α-Fodrin is located at the inner surface of the cell membrane, at the T tubuli and the Z lines, and it has also been described in the intercalated disks in the myocardium. Desmin has been associated with the Z lines and with other cytoskeletal elements. The degradation of these structural proteins could possibly affect the stability of the sarcomeres and hence the longitudinal transmission of force, and/or decrease the efficacy of cross-bridge cycling. With regard to previous animal experiments, where a high level of α-actinin loss was detected from postischemic hearts, it is somewhat

surprising that human α-actinin was the least susceptible of all the proteins investigated to μ-calpain proteolysis. A possible explanation for this was provided by Goll et al., who suggested that μ-calpain may release α-actinin from the Z line (without degrading it) via the modulation of other interacting proteins. Thus, the loss of Z line α-actinin does not necessarily correspond to the degradation of the molecule, but rather to its release from a loose structure during postischemic stunning. The μ-calpain proteolysis of the regulatory cTnT under our experimental conditions was relatively rapid. Unfortunately, the extent to which cTnT degradation is involved in the altered Ca^{2+} reactivity of the myofibrillar system in the postischemic myocardium is unknown. Similarly as for cTnI, no appreciable difference in μ-calpain susceptibility between the donor and the patient hearts was detected for all the other investigated proteins. This finding implies that postischemic myofibrillar protein degradation may develop similarly in the healthy and the remodeled, dilated myocardia.

Endogenous Ca^{2+}-dependent proteolytic activity in the human myocardium

The endogenous Ca^{2+}-activated proteolytic alterations were reminiscent of those induced by *in vitro* test incubations with μ-calpain. Additionally, the differences between the investigated proteins as concerns their susceptibilities to undergo this intrinsic degradation were also comparable with what we demonstrated for exogenous μ-calpain. These data imply that the human myocardium has the potential to modulate its myofibrillar proteins via its own calpains. It is also worthy of consideration that cTnI was less susceptible than titin, α-fodrin, desmin or cTnT to μ-calpain proteolysis. Hence, degradation of all the above proteins is expected to parallel the myofibrillar dysfunction during transient μ-calpain activation when cTnI proteolysis occurs. Our data are therefore in line with the view that μ-calpain-mediated proteolysis is not likely to be restricted to merely a single protein, but rather represents a major pathway of post-translational modification.

Summary

The potential role of μ-calpain in the development of familial hypertrophic cardiomyopathy and myocardial stunning was investigated in the present study.

On the basis of μ-calpain-sensitivity analysis of purified recombinant wild-type cTnI and three of its fHCM-related missense mutants (R145G, G203S and K206Q) and human myofibrillar proteins (titin, α-fodrin, desmin, cTnT, cTnI and α-actinin) we came to the following conclusions:

1. μ-calpain-susceptibility of both the wild-type and the mutant cTnI molecules were similar *in vitro*. This suggested that mutations in the central inhibitory domain (R145G) and in the C-terminal region (G203S and K206Q) of cTnI do not affect its μ-calpain-mediated degradation. Therefore, altered μ-calpain-mediated cTnI proteolysis is not likely to play a role in the pathomechanism of fHCM.
2. The degradation of the cTnI molecules in the troponin complex was less intense than that of the noncomplexed forms.
3. Phosphorylation by protein kinase-A conferred effective protection against cTnI proteolysis in recombinant molecules, however this phosphorylation-mediated protection was not observed in donor hearts despite their higher cTnI phosphorylation level.
4. We identified several myofibrillar proteins in the human myocardium to be sensitive to μ-calpain, in addition to cTnI. Titin and α-fodrin exhibited high, desmin and cTnT moderate and cTnI and α-actinin low μ-calpain susceptibility. The level of phosphorylation of cTnI did not explain its relatively low μ-calpain susceptibility. Moreover, the molecular mass distributions of the truncated α-fodrin, desmin and cTnI fragments resulting from Ca^{2+}-dependent autoproteolysis exhibited marked similarities with those of their μ-calpain-clipped products. These *in vitro* results shed light on a number of structural (titin, α-fodrin, desmin and α-actinin) and regulatory (cTnT and cTnI) proteins within the contractile apparatus as potential targets of μ-calpain. Their degradation may contribute to the development of postischemic stunning in the human myocardium.

Publications related to Thesis

***In extenso* publications:**

Barta J, Tóth A, Jaquet K, Redlich A, Édes I, Papp Z: Calpain-1-dependent degradation of Troponin I mutants found in familial hypertrophic cardiomyopathy. **Mol Cell Biochem** 251: 83-88, 2003.
IF.: 1.548

Barta J, Tóth A, Édes I, Vaszily M, Papp JG, Varró A, Papp Z: Calpain-1 sensitive myofibrillar proteins of the human myocardium. **Mol Cell Biochem** (in press) 2004.
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