SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Myofilament protein alterations resulting in contractile changes of cardiomyocytes

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The Examination will be held 9:00 AM, February 16, 2021. (online)

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1. Introduction and background

There is an important, close interaction between the pathomechanism of cardiovascular disease and oxidative stress. In cardiomyocytes, an imbalance between ROS formation (also produced under normal physiological conditions) and antioxidants leads to an alteration of cellular oxidoreductive status. Clinical studies have made the role of ROS indisputable in the development and progression of heart failure. Free radicals induce direct damage of contractile proteins resulting in cardial dysfunction. In recent years, the giant protein, titin has received special attention among intracellular elements and its indirect (e.g. via heat shock proteins) and direct (e.g. caused by oxidation) alterations play a key role in the adaptive and pathological changes in diastolic stiffness.

It is well known that the heart reacts to pathological stimuli (e.g. increased pressure overload) through adaptive processes which contribute to hypertrophic remodeling and the development of pathological cardiac hypertrophy. Alterations of various signaling pathways have been shown to underlie the above process. It is not surprising that significant efforts have recently been made in the field of basic research to develop *in vivo* animal models contributing to thebetter understanding of the pathophysiology of remodeling induced by cardiac hemodynamic overload.

1.1. Structure and mechanics of the sarcomere

In cardiomyocytes thick (6-9 nm in diameter) and thin (12-15 nm in diameter) filaments are organised in sarcomers which are capable of generating contractile force. Thin filaments, consists primarily of the contractile protein, actin and the regulatory troponin (Tn)- tropomyosin (Tm) complexes in which the double helix structure formed by polymeric actin fibers twist around each other. The characteristic phosporylation sites of cardiac toponin I in case of protein kinase A-mediated sites, are serin 22/23 (Ser-22/23) in rodents, while Ser-23/24 in the human myocardium, protein kinase C-mediated sites are Ser-43/45 and threonin-144 (Thr-144). Thick

filaments, located in the middle of the sarcomere, are composed of myosin light chains (MLC) and heavy chains (MHC) and organized in head and tail domains. The 140 kDa cardiac myosin-binding protein C (cMyBP-C), which is associated with actin, myosin, and titin and has a crucial role in the actin-myosin interaction, is involved in the structure of the thick filament. The cMyBP-C protein has three serine residues (Ser-273, Ser-282 and Ser-302) avalaible for phosphorylation. Overall, posttranslational modifications of proteins with structural and regulatory functions (as constituents of myofilaments) can affect both force generation efficiency and Ca^{2+}-sensitivity of cardiomyocytes.

Beyond their active mechanical function, striated muscles have a very important feature, the flexibility. Without disrupting the structural order of the sarcomere, muscle fibers can be stretched to more than twice of their original length. During this process, the so-called passive force (resistance) develops, which restores the resting length of the muscle after the termination of the stretching force. The third filamentary system of the sarcomere, especially its titin component, is responsible for the passive elasticity of the muscle.

1.2. The location and structure of titin

Titin is the major determinant of cellular diastole and passive tension of cardiomyoctyes. The elongated length of this molecular spring is approximately 1 μm, titin spans half of the sarcomere from Z-disk to M-line. Its isoform-dependent molecular size is ~3000-3800 kDa (in comparison, actin has a molecular weight of 43-48 kDa and myosin has a molecular weight of 480 kDa).

Z1 and Z2 domains of titin molecules are cross-linked to Z-disk by telethonin (also known as T-cap/titin-cap) and α-actinin. 800-1500 kDa parts of titin are located in the I-band region where they form a flexible connection between the end of the thin filaments close to Z-disk and the peak regions of thick filaments. A 2 MDa part of the molecule is located in the A-band, where six titin molecules bind to each half of thick filaments thereby forming a close

relationship with both myosin and cMyBP-C proteins. Titin is encoded by a single gene (TTN) of 363 exons. Despite of its giant size it has a relatively monotonous structure. *The primary structure of titin* consists of an average of 27000-33000 amino acids per isoform, and according to current databases, the total protein may consists of 38138 amino acids. *Secondary structure*. The sequence of titin consists of multiple repeats of two different types of 90-100 amino acids. Later, the first type was classified into a superfamily of type I immunoglobulin (Ig) domains, and the second type into a superfamily of fibronectin type III (Fn) domains. Besides the domains approximetly 8-10% of titin are unique sequences. From functional point of view, the most important is the PEVK segment in I-band region, is named after the amino acids proline (P), glutamic acid (E), valine (V) and lysine (K), contributing to its structure. *Under the tertiary structure of titin*, series of domains and individual sequences are meant, their relationship being a function of the sarcomere state and other (patho) physiological states.

1.2.1. The function of titin

The primary function of titin is to provide passive muscle elasticity according to physiological needs, based on different isoforms of the molecule.

Titin is also expressed in isoforms that differ in size and elasticity. In adult human heart, a shorter and stiffer N2B (~3000 kDa, 70% of total titin) and a longer, more elastic N2BA (~3800 kDa, 30% of total titin) isoforms are expressed. As a result, shorter isoforms result higher, longer titin isoforms result less passive tension. Composition of titin isoform varies by species and ages. In newborn rat cardiomyocytes the more compliant N2BA isoform (approx. 90%) is the dominant, however, the expression of the smaller N2B isoform is increased after birth (approx. 90-95%), enhancing passive tension in adult cardiomyocytes. Titin-based passive elasticity of cardiomyocytes is not a conserved feature, thus changes in the isoform composition of titin (e.g. with age), the oxidative and phosphorylation status and other physiological factors

(e.g. titin-bound heat shock proteins) can affect the cardiomyocytes passive tension by directly and indirectly.

1.3. Direct modification of titin-dependent passive stiffness: Oxidative stress

Ratio of free radicals and antioxidants is determinant for normal tissue function, disruption of this balance can contribute to the generation of oxidative damage, which plays a significant role in the development of many cardiovascular diseases. Overproduction of free radicals is counterbalanced by the body's antioxidant system, but the imbalance causes the formation of reactive oxygen / nitrogen species (ROS / RNA) and leads to oxidative stress conditions. Among amino acids making up proteins, cysteine is extremely sensitive to oxidation. Due to oxidation, proteins of cardiomyocytes can suffer reversible and irreversible damages are highlighted as sulfhydryl (SH) oxidation and carbonylation.

1.3.1. Oxidation of sulfhydryl groups

Reversible oxidation of sulfhydryl (SH)-groups presents on the cysteine side chains of myocardial proteins has a significant physiological and pathophysiological role in eukaryotic cells. During oxidative stress, the modification of SH groups in proteins is thought to occur among the first, thereby play an essential role in cell protection. Owing to its reversible oxidation, it protects proteins from irreversible modification. Reversion of the disulfides formed during oxidation can be achieved with reducing molecules.

Previous experiments in our laboratory have demonstrated that SH-oxidation of contractile proteins may contribute to the development of contractile dysfunction during heart failure. It has been shown that the SH-group specific *in vitro* oxidative agent, dithiodipyridine (DTDP, 2.5 mM) declined the Ca^{2+} -dependent active force and Ca^{2+} -sensitivity, and slightly enhanced Ca^{2+} -independent passive tension in cardiomyocytes.

It has also been hypothesized that the oxidation of titin molecule may have an effect on passive tension, however, a more detailed study of titin oxidation was not possible, due to the

lack of literature data and technical difficulties related to the gigantic molecular weight of the protein. These factors provided the starting point to this thesis.

Six cysteine molecules have been shown in the N2B region (N2-Bus) of titin containing unique sequences, are able to form three disulfide bonds as a result of oxidation, all of which significantly increase passive stiffness. Therefore, SH oxidation of this titin region has also proven to be pathologically significant.

1.3.2. Carbonylation

Protein carbonylation - the commonly considered biomarker of oxidative stress may also bear with functional consequence on sarcomeric funtion. Protein carbonylation of lysine, arginine, proline or threonine residues develops in response to a hydroxyl radical mediated reaction involving reactive aldehydes (or ketones), through the participation of hydrogen peroxide (H_2O_2) and iron (II) (i.e. Fenton reaction). Fenton reaction is widely used to study protein carbonylation *in vitro*. Nevertheless, no information is available on how titin carbonylation modulates the titin-dependent passive force of cardiomyocytes.

1.4. Indirect modification of titin-dependent passive stiffness: heat shock proteins

Small heat shock proteins (sHSP) such as HSP27 (HSPB1) and αB-crystallin (HSPB5) are produced in large amounts primarily in heart muscle, skeletal muscle, kidney, and brain. Expression of these two proteins are increased in response to stress effects.

HSP27 and αB-crystallin heat shock proteins provide protection against oxidative damage. On the one hand they regulate the amount of reduced glutathione in oxidative protection, and the other hand decrease the amount of ROS / RNA radicals, and thus the rate of protein oxidation. During stress effects, they preferentially translocate to the sarcomere, the exact mechanism of it is still unclear. HSP27 and αB-crystallin are known to be induced by ischemia, heat stress or heart failure.

Under stress, sHSPs pass through from cytosol to the intracellular space, where they are bound to Z-disk and / or I-band in the sarcomere.

Due to the sHSPs-interaction, sarcomeric proteins such as actin, desmin, myosin, tropinin-I, troponin-T and titin belong to protected proteins.

However, under pathological conditions, sHSPs are translocated not only to Z-disk but also to the functionally elastic I-band region of the titin molecule, thereby regulating passive stiffness of cardiomyocytes. *In vitro* stabilization of Ig domains (proximal, middle and distal) and N2-Bus domains in this molecularly elastic region of titin is mediated through the binding of sHSP proteins. Experiments demonstrated that cryptic hydrophobic sites surfaced during the unfolding of titin domains, are remained protected from oxidation by sHSPs during oxidative stress. According to these, research suggests that interaction between sHSPs and titin molecule is enhanced by both stretching of the sarcomere and the expression of the more rigid N2B isoform, thus protecting titin domains from possible aggregation. Studies over the past decade propose that function of HSPs is greatly influenced by age in addition to oxidative stress.

However, based on current data, it is not known how much small heat shock proteins (HSP27 and αB-crystalline) bound to titin under physiological conditions that may affect the extent of in vitro oxidative titin-dependent passive tension in cardiomyocytes of various developmental stages.

1.5. Compensated and decompensated pathological left ventricle-hypertrophy

Chronic pressure overload of the heart results in increased work leading to heart failure. Initially, left ventricle adapts to the altered hemodynamic conditions with compensatory hypertrophy to maintain adequate pressure. All of this supports circulation in the short term, however, hypertrophy is detrimental in the longer term. Compensatory mechanisms on the long term lead to structural and functional remodeling of the myocardium, resulting in insufficient ventricular filling and emptying, as well as extracellular matrix fibrosis, myocardial cell necrosis, and apoptosis, leading to contractile dysfunction. It is not yet fully clarified exactly how adaptive hypertrophy is transformed into maladaptive hypertrophy.

Characteristic changes can be observed in the contractility of so-called early (compensated) and advanced (decompensated) stages of pathologic left ventricular hypertrophy.

Experimental work with pressure-volume analysis demonstrates an increase in contractility in the early stages of left ventricular hypertrophy, during which the heart compensates for increased afterload, thus ensuring the preserved systolic function of the heart. However, all this allows only temporary adaptation, in more advanced, late stages, contractility parameters deteriorate significantly, systolic and diastolic performance decrease. In addition, progressive collagen accumulation and the appearance of other fibrotic markers can be observed.

Research anticipates that during chronic pressure overload, left ventricular contractility causes changes not only in the organ but also in the sarcomeric function. However, no information is available on stage-dependent changes in the function of myofilament system of cardiomyocytes. Since left ventricular remodeling contributes to contractile dysfunction over a long period of time, it has been suggested that processes from compensated phase to decompensated phase may also serve as therapeutic targets.

2. Aims

2.1. Titin-based passive tension and the oxidative modifications of titin isoforms during cardiomyocytes developement

We aimed to investigate the oxidation of titin isoforms (SH oxidation, carbonylation) on titin-based passive tension in different stages of cardiomyocyte developement in permeabilized cardiomyocytes. To this end, we examined the effects of oxidative agents (DTDP, Fenton reagents) on the mechanical function of cardiomyocytes isolated from different age groups and determined the oxidative sensitivities of titin isoforms in parallel.

Moreover, we investigated the expression level of titin-associated small heat shock proteins (HSP27 amd αB-crystallin) during cardiomyocytes maturation and examined their probably role in the regulation of titin protein oxidation.

2.2. Myofilament system of early and advanced pathological hypertrophy in pressure overload-induced rat model

We aimed to invastigate the functional and structural alterations of early/compensated and advanced/decompensated pathological miocardium hypertrophy in permeabilized cardiomyocytes. We examined the mechanical performance of isolated cardiomyocytes and identified the phosphorylation status of myofilament proteins (cardiac troponin I and cardiac MyBP-C).

3. Materials and methods

3.1. Methods I: Rat model of oxidative examinations

Experimental cardiac muscle specimens

Myocardial samples used in our oxidation experiments were collected from 0-, 7-, 21-day-old and adult (8 week-old) healthy control Wistar-Kyoto rats. All procedures were approved by the Ethical Committee of the University of Debrecen (Ethical Statement No. 24/2013/DE MÁB).

Titin-based passive tension measurements in permeabilized cardiomyocytes

Left ventricular (LV) myocardium derived from rats of different ages (0-, 7-, 21-day-old, and 8-week-old adults) were mechanically disrupted in isolated solution and thereafter permeabilized. The titin-based passive tension ($F_{passive}$) of membrane-deprived cardiomyocytes obtained in this way were measured on a custom-developed myocyte set-up contractile system of our Division. Single permeabilized cardiomyocytes were mounted with silicone adhesive (DAP 100% all-purpose silicone sealant; Baltimore, MD, USA) to the central unit of the set-up system. The sarcomere length (SL) dependence of $F_{passive}$ of cardiomyocytes of different ages

was examined by gradual stretching of the cells (SL: 1.9–2.5 μm), while the detection of oxido-reductive effects was measured in physiological SL (2.3 μm) under video-microscope control. Measurements were performed in Ca^{2+} -free relaxing solution, where the Ca^{2+}-independent $F_{passive}$ was approximated by shortening of the preparations to 80% of the original lengths for 8 sec. $F_{passive}$ was normalized for cardiomyocyte cross-sectional area and values are expressed in absolute force units (kN/m^2).

To modulate the reduced/oxidated SH-group content or carbonylation status of titin isoforms isolated cardiomyocytes were incubated at room temperature in relaxing solution supplemented with DTDP (10 mM) or Fenton reagents (50 μM $FeSO_4$, 1.5 mM H_2O_2, 6 mM ascorbic acid). Reversibility of the oxidative effects was tested by sequential application of thiol-groups reductant DTT (10 mM, Sigma-Aldrich, St. Louis, MO, USA) for 30 min.

Determination of SH group oxidation in titin isoforms

Cardiomyocytes at different ages were treated in relaxing solution containing DTDP (10 mM, for 2 min) or DTT (10 mM, for 30 min). Reduced/free SH-groups of titin isoforms of cardiomyocytes were labeled by EZ-Link Iodoacetyl-LC-Biotin in a reaction buffer according to the manufacturer's instructions. After biotinylation, proteins of permeabilized cardiomyocytes were solubilized in sample buffer. Protein concentration was adjusted uniformly to 2 mg/ml in every case. After agarose-strengthened 2% SDS-polyacrilamide gels titin isoforms were transferred onto an Immobilon-FL PVDF membrane. Biotin-labeled SH-groups were probed with phycoerytrin-conjugated (PE) streptavidin. Total protein amounts were quantitated with the super sensitive blot staining.

Determination of titin isoforms carbonylation by oxyblot assay

The carbonyl group content of titin isoforms was determined using an oxyblot protein detection kit. Permeabilized cardiomyocytes were treated in relaxing solution containing Fenton reagents. Cardiomyocytes were solubilized in sample buffer and the carbonyl groups

were derivatized through a reaction with 2,4-dinitrophenylhydrazine (DNPH). After gel electrophoresis proteins were transferred onto Immobilon-FL PVDF membrane. After blocking the membranes labeling with the following antibodies were applied: primary (rabbit anti-DNPH antibody 1:150, 1 h) and biotin conjugated secondary antibodies (goat anti-rabbit IgG 1:1000 45 min). Biotin-labeled carbonyl-groups were probed with phycoerytrin-conjugated (PE) streptavidin. Titin isoforms were visualized with a super sensitive membrane stain. Data are given relative to signal intensities of untreated controls (carbonylation index, CI).

Western immunoblot analysis of HSP27 and αB-crystallin expression

After isolation and permeabilization LV cardiomyocytes were dissolved in SDS sample buffer. Proteins were separated by 8% SDS-polyacrylamide gels then transferred onto nitrocellulose membrane. Membranes were probed with primary [(anti-HSP27 (1:300) or anti-αB-crystallin (1:1000)] and secondary antibodies [(peroxidase- conjugated goat anti-mouse (1:100000) or goat anti-rabbit (1:300)]. Total protein amounts were quantified with super sensitive membrane stain.

Immunohistochemistry

Cardiomyocytes were isolated from frozen LV heart tissues the same way as for the functional measurements. Permeabilized cardiomyocytes were fixed on microscope slides. They were incubated with primary antibodies [(anti-HSP27 (1:50) anti-αB-crystallin (1:300) and anti-titin (1:50)] and thereafter secondary antibodies were applied [goat-anti-rabbit Cy2 and Cy3 and biotinylated goat-anti-mouse (1:200)]. Biotinylated antiobodies were detected by Cy2- and Cy3- conjugated streptavidine (1:500). Images were captured by a Scion Corporation digital camera attached to a Nikon Eclipse 80i fluorescent microscope.

3.2. Methods II: Animal model of left ventricule hypertrophy

Experimental cardiac muscle specimens

The animal model of pressure overload-induced pathological hypertrophy was set up and characterized by the colleagues of Semmelweis University Heart and Vascular Center. These investigations were performed in accordance with the Guide for the Care and Use of Laboratory Animals (US National Institues of Health 1996; 85–23) and approved by the appropriate ethics review committee of Baden-Württemberg (license code number: G-94/15).

Abdominal aortic banding and experimental animal groups

At the end of the acclimatization period (1 week), animals underwent Sham surgery (Sham / control) or surgical constricting of the abdominal aorta (abdominal aortic-banding-AB). During AB surgery (n=39), abdominal aorta was constricted to the same size as the external diameter of a 22-gauge needle. Except the aortic constriction, Sham-operated animals (n=28) were subjected to the same surgical procedure. Following surgery, to relieve postoperative pain buprenorphine was provided in the dose of 0.05 mg/kg.

Experimental Groups

After the aortic banding or sham operation, animals were divided into 6 experimental groups: (1) Sham-6 weeks (n=9): 6-wk-long follow-up period after sham operation, (2) AB-6 weeks (n=13): 6-wk-long follow-up period after aortic banding procedure, (3) Sham-12 weeks (n=9): 12-wk-long follow-up period after sham operation, (4) AB-12 weeks (n=13): 12-wk-long follow-up period after aortic banding procedure, (5) Sham-18 weeks (n=10): 18-wk-long follow-up period after sham operation, (6) AB-18 weeks (n=13): 18-wk-long follow-up period after aortic banding procedure.

Cardiomyocytes force measurements

Deep-frozen LV tissue samples were mechanically disrupted, permeabilized and fixed to the contractile system as previously described. Isometric forces were measured uniformly at an adjusted sarcomere length of 2.3 μm. Ca^{2+}-dependent isometric forces (F_{active}) of

cardiomyocytes were measured by transferring the preparation from the relaxing to the activating solution. Maximal Ca^{2+}-dependent active force (F_{max}) and Ca^{2+}-sensitivity (pCa_{50}) of cardiomyocytes were recorded at maximal (pCa = 4.75) and submaximal (pCa = 5.2-7.0) Ca^{2+} concentrations. Submaximal pCa-evoked active force values were normalized to maximal force (pCa 4.75) and fitted with a special sigmoid function (modified Hill equation) using the Origin 6.0 analysis program. From the so-called Ca^{2+}-sensitivity curve thus obtained, pCa value required for half-maximal exertion (pCa_{50}) can be derived, which alone gives the Ca^{2+}-sensitivity of cardiomyocytes. Ca^{2+}-independent $F_{passive}$ of cardiomyocytes in relaxing solution was performed as previously described.

Determination of the phosphorylation status of cardiac TnI and MyBP-C proteins

LV cardiomyocytes were isolated and permeabilized as in mechanical measurements and then dissolved in SDS sample buffer. Protein concentrations were determined by dot-blot method with bovine serum albumin (BSA) standard solution series and adjusted uniformly to 2 mg/ml. Separation of cardiac troponin I (cTnI) and cardiac myosin-binding protein C (cMyBP-C) was performed using 12% and 4% polyacrylamide gels and proteins were identified by protein standard. Following gel electrophoresis, samples were transferred to a nitrocellulose membrane and after blocking, proteins were labeled with primary antibodies against at the cTnI phosphorylated site of PKA-dependent Ser-22/23, PKC-dependent Ser-43 and Thr-144, and at cMyBP-C phosphorylated site of PKA-mediated Ser-282 [(anti-cTnI Ser-23/24 (1:1000), anti-cTnI Ser-43 (1:500) and anti-cTnI Thr-144 (1:500) and anti-cMyBP-C Ser-282 (1:500)]. Signals were detected using peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:300). Total protein amounts were quantified with the super sensitive membrane stain. Chemiluminescent signals (ECL) were normalized to total protein.

Statistics

Statistical significance was tested by analysis of variance (ANOVA followed by Bonferroni's post hoc test). Values are given as mean ± SEM. The limit for statistical significance was $P < 0.05$.

4. Results

4.1. Titin-dependent passive tension and the oxidative modifications of titin isoforms during cardiomyocytes maturation

In our initial experiments, we were able to confirm previous observations that perinatal adaptation to extrauterine life involves a titin isoform switch resulting in a gradual increase in the expression of the less compliant N2B isoform - with unchanged titin total protein- in expense on a gradual decline in the expression of the more compliant N2BA isoform leading to an increase in Ca^{2+} -independent $F_{passive}$ in cardiomyocytes with age. A complete reproduction of previous literature data suggested that our experimental method is appropriate achieving our objectives despite the technical difficulties due to the giant size of titin protein and the extremely small left ventricular mass of the neonatal rat heart.

4.1.1. SH oxidation of titin isoforms increases $F_{passive}$ in an age-dependent manner

Following exposures to the SH-oxidant DTDP $F_{passive}$ increased significantly in all age-groups, but to relatively higher extents in the 0-day-old and 7-day-old age groups than in the 21-day-old and adult age groups ($F_{passive}$ changed from 0.09±0.02 kN/m^2 to 0.18±0.03 kN/m^2 in the newborn group; from 0.19±0.02 kN/m^2 to 0.31±0.04 kN/m^2 in the 7 day-old age group; from 0.45±0.07 kN/m^2 to 0.60±0.07 kN/m^2 in the 21 day-old group and from 2.35±0.11 kN/m^2 to 2.76±0.16 kN/m^2 in cardiomyocytes of adult rats, SL: 2.3 μm, $P<0.05$ *vs.* control in all age groups, n=6-8 cardiomyocytes from at least three different hearts). DTT was applied after DTDP treatments to test the reversibility of SH oxidation. DTDP mediated increases in $F_{passive}$ were reversed upon DTT treatment in all age groups ($F_{passive}$ following DTT were: 0.10±0.01 kN/m^2; 0.21±0.02 kN/m^2; 0.51±0.08 kN/m^2 and 2.28±0.12 kN/m^2 in 0, 7, 21 day-old and adult

rat cardiomyocytes, respectively, SL: 2.3 μm; n=6-8 cardiomyocytes from at least three different hearts). A biotinylation-based assay was included to evaluate the SH oxidative changes of N2BA and N2B titin isoforms following DTDP exposures. DTDP decreased the free SH-group content of N2BA and N2B titin isoforms by similar extents (normalized SH-group content: 100% before DTDP exposures; control) in permeabilized cardiomyocytes of the 0-, 7-, 21-day-old and adult age groups (*i.e.* by: 80±1%, 71±1%, 64±1% and NA in the N2BA titin isoform; and by: 74±1%, 62±2%, 53±2% and 32±2% in the N2B titin isoform; $P<0.05$ *vs.* control, for the above age groups, respectively, n=8-23 independent determinations). The free SH-group content of N2BA titin isform in the adult age group could not be assessed due to the low expression level of this titin isoform. Free SH-group sensitive signal intensities returned to control levels following DTT applications, moreover, DTT alone had no effect on cardiomyocyte SH-group oxidation.

4.1.2. Carbonylation of titin isoforms increases $F_{passive}$ in an age-dependent manner

The effects of protein carbonylation on $F_{passive}$ were investigated in permeabilized LV cardiomyocytes in different age groups following incubations in the presence of Fenton reagents. Similarly as above, attempts were made to reverse the Fenton effects by the application of DTT. Fenton reagents increased $F_{passive}$ significantly in 0-, 7-, 21-day-old and adult groups, but gradually to less and less relative extents with cardiomyocyte age (*i.e.* to: from 0.11±0.01 kN/m^2 to 0.22±0.01 kN/m^2 in 0-day-old, from 0.26±0.02 kN/m^2 to 0.44±0.05 kN/m^2 in 7-day-old, from 0.49±0.03 kN/m^2 to 0.69±0.06 kN/m^2 in 21-day-old, from 2.17±0.05 kN/m^2 to 2.59±0.10 kN/m^2 in adult rats, respectively, $P<0.05$ *vs.* control, n=6-7 cardiomyocytes from at least three different hearts). Moreover, the above mechnical effects were fully reversed by the application of DTT in the 0-,7- and 21-day-old groups, but not in adult group ($F_{passive}$: 0.11±0.01 kN/m^2; 0.22±0.02 kN/m^2; 0.35±0.03 kN/m^2 and 2.94±0.15 kN/m^2, in the above age groups, respectively, $P<0.05$ *vs.* control, n=6-7 cardiomyocytes from at least three different

hearts). In paralell biochemical investigations oxyblot assays were performed to reveal the extents of carbonyl group formations of N2BA and N2B titin isoforms in myocardial tissue homogenates following incubations in the presence of Fenton reagents. The levels of carbonyl groups increased significantly following exposures to Fenton reagents, although in an apparent negative correlation with age (*i.e.* to 2.57±0.06 AU, 2.37±0.02 AU, 1.35±0.03 AU, in 0-, 7-, 21-day-old cardiomyocytes, respectively, in N2BA titin isoforms; and to: 2.90±0.10 AU, 2.57±0.04 AU, 1.79±0.03 AU, 1.51±0.02 AU, in 0-, 7-, 21-day-old and adult cardiomyocytes, respectively, in N2B titin isoforms, $P<0.05$, n= 9-18 independent determinations from at least three different hearts). Protein carbonylation of the N2BA titin isform in the adult age group could not be assessed due to the low expression level of this titin isoform. Titin carbonylations were generarly reversed by DTT, except in adult cardiomyocytes.

4.1.3. Gradual increases in the expression levels of HSP27 and αB-crystallin proteins in developing cardiomyocytes

To characterize the levels of HSP27 and αB-crystallin expressions in permeabilized cardiomyocytes tissue homogenates from 0-, 7-, 21-day-old and adult rat hearts were analyzed by Western immunoblotting. These investigations revealed, that the relative expression levels of HSP27 (1.04±0.08 AU, 2.14±0.30 AU, 3.04±0.49 AU, 4.83±0.54 AU in 0-, 7-, 21-day-old and adult rats, respectively, $P<0.05$ *vs.* day 0 (d0, reference level:1), n=9-17 determinations from at least three different hearts), and αB-crystallin (1.00±0.11 AU, 1.61±0.19 AU, 3.23±0.86 AU, 4.42±0.96 AU in 0-, 7-, 21-day-old and adult rats, respectively, $P<0.05$ *vs.* d0, n=8-15 determinations from at least three different hearts) gradually increased with cardiomyocyte age. In accordance with the biochemical data, immunohistochemical staining of LV cardiomyocytes revealed progressive HSP27 and αB-crystallin expressions with remarkable colocalizations with titin proteins during cardiomyocyte maturation.

4.1.4. Correlations between oxidative changes of cardiomyocyte $F_{passive}$, oxidative changes of titin isoforms and expression levels of HSP27 and αB-crystallin during cardiomyocyte development

Strong negative correlations were noted when protein oxidation evoked changes in $F_{passive}$, oxidized SH contents or carbonylation indices of titin isoforms were contrasted to the expression levels of HSP27 or αB-crystallin at different cardiomyocyte ages.

4.2. Investigation of myofilamental changes in a rat model of pressure overload-induced pathological hypertrophy

We aimed to study the relation between contractile parameters of left ventricule (F_{max}, pCa_{50} and $F_{passive}$) and myofilament function (cTnI and cMyBP-C) during the development and progression of pressure overload-induced myocardial hypertrophy.

4.2.1. The maximal Ca^{2+}-activated force generation unchanged in different stage of pressure overload-induced left ventricular hypertrophy

The Ca^{2+}-dependent force generation was investigated on left ventricular cardiomyocytes from Sham and AB animals after 6-, 12-, and 18-week aortic-banding. No significant differences could be observed in F_{max} among the control experimental groups (6 weeks: 15.32±0.78 kN/m^2; 12 weeks: 14.19±0.47 kN/m^2; 18 weeks: 13.62±0.79 kN/m^2, n=9-10 cardiomyocytes/group) and AB (6 weeks: 13.57±0.69 kN/m^2; 12 weeks: 13.01±0.76 kN/m^2; 18 weeks: 15.49±1.02 kN/m^2, $P<0.05$, n=13 cardiomyocytes/group).

4.2.2. The Ca^{2+}-sensitivity of cardiomyocytes increased in early stage of pressure overload-induced left ventricular hypertrophy

The pCa_{50} increased significantly in the AB-6 weeks (5.88±0.02, $P<0.05$) group compared to the Sham-6 weeks group (5.73±0.01), indicating increased myofilament Ca^{2+}-sensitivity. This pCa_{50} value was also significantly higher in the AB-6 weeks group than in the AB-12 weeks (5.77±0.01) and AB-18 weeks groups (5.74±0.01). Myofilament Ca^{2+} -sensitivity in the AB-12 weeks and AB-18 weeks groups did not differ from their

corresponding control groups (Sham-12 weeks: 5.75±0.01 and Sham-18 weeks: 5.74±0.02).

4.2.3. Titin-dependent passive tension unchanged the development of pathological hypertrophy in the rat model

Ca^{2+} -independent passive tension of permeabilized myocardial cells did not change in the early (AB-6 weeks: 1.34±0.01 kN /m^2) and advanced (AB-12 weeks: 1.09±0.04 kN/m^2 and AB-wk18: 1.05±0.09 kN/m^2, n= 9-13 cardiomyocytes/group) pathological left ventricular hypertrophy compared to the corresponding Sham groups (Sham-6week: 1.07±0.08 kN/m^2; Sham-12 weeks: 1.14±0.07 kN/m^2 and Sham-18 weeks: 1.15±0.14 kN/m^2).

4.2.4. Site-specific phosphorylation of cTnI and cMyBP-C

The PKA and PKG site-specific phosphorylation of cTn-I and cMyBP-C proteins were investigated by Western-immunoblot technique. Phosphorylation of PKA-specific cTnI at Ser-22/23 site and Ser-43 site did not differ among the study groups. In contrast, phosphorylation of cTnI at the PKC-specific Thr-144 site was significantly increased in the AB-6 weeks group (1.51±0.09) compared either to the Sham-6 weeks group (0.98±0.04, *P<0.05), or the other Sham and AB groups (Sham-12 weeks and 18 weeks: 1.00±0.04 and 1.00±0.05; AB-12 weeks and 18 weeks: 1.07±0.09 and 0.91±0.04, n=3 different heart/group, *P<0.05). There was also no difference in the phosphorylation status of cMyBP-C at Ser-282.

5. Discussion

Titin isoforms are increasingly protected against oxidative modifications in developing rat cardiomyocytes

In our study, we characterized for the first time the effect of titin protein oxidation on passive tension during postnatal adaptation of the heart. Major novelties of the present study are that titin oxidation and carbonylation have similar effects on N2BA and N2B titin isoforms, and myofibrillum associated heat shock proteins (HSP27 and αB-crystallin) may confer

protection against the above forms of oxidative modifications. Interestingly, this putative HSP-mediated protection builds up gradually with postnatal cardiomyocyte age, without the influence of experimental stress of any forms.

In this study, $F_{passive}$ was probed by exposures to oxidative agents in isolated, permeabilized cardiomyocytes at physiologically relevant sarcomere lengths and at various postnatal developmental stages. This approach also allowed the investigation of a hypothetical link between titin isoform oxidation and sHSPs expression. In our present work, SH-group oxidation of titin isoforms was provoked by an SH-group specific oxidative agent, DTDP *in vitro*, which was prevented by the application of SH-group reduction agent, DTT *in vitro* in maturating cardiomyocytes of rats. We observed that the DTDP evoked increments in $F_{passive}$ declined with age in permeabilized cardiomyocytes of the rat in a reversible fashion. Moreover, the extent of DTDP evoked SH-group oxidation also declined with age in both N2BA and N2B titin isoforms.

In addition to SH-group oxidation, ROS induced myofilament protein carbonylation can also contribute to contractile dysfunction in the heart. Hydroxyl radicals are highly reactive and thus react with divergent cell components. In this study, Fenton reagents were used to produce hydroxyl radicals and to induce titin carbonylation *in vitro*. Fenton reagents increased $F_{passive}$ in all age groups. Moreover, similarly to the effects of DTDP, the increases in $F_{passive}$ declined with age. Furthermore, the extent of titin carbonylation also diminished with age.

Taken together, for oxidative challenges of identical strengths, the extents of titin SH oxidations or carbonylations declined with age for both titin isoforms. This effect apparently did not depend on the N2B/N2BA titin isoform expression ratio. Moreover, these findings are also suggestive for a gradual decline in the oxidative sensitivities of cardiomyocyte $F_{passive}$ with cardiomyocyte maturation, which was parallel to the extent of SH oxidation or carbonylation of titin isoforms in developing heart.

Previous experimental studies furnished with evidence on the protective roles for sHSPs for sarcomeric proteins such as actin, desmin, myosin, troponin-I, troponin-T and titin. sHSPs have been implicated in promoting physiological protein folding and preventing misfolding, thereby protecting the function of native macromolecules, and limiting the effects of oxidative stress in general. In the above context misfolded proteins have been associated with higher sensitivities for carbonylation than those of their native forms. Two myocardial sHSPs, HSP27 and αB-crystallin are known to be induced by heat stress, ageing, end-stage heart failure or ishemic injury. Moreover, these proteins are also known to be involved in preventing the effects of oxidative stress and in the regulation of cellular redox states. HSP27 and αB-crystallin can both colocalize with I-band titin in cardiac and skeletal muscles, translocate and bind to the spring elements of titin isoforms under stress conditions. A potential contribution of sHSPs to normal passive elasticity and a role in the maintenance of normal function of cardiac and skeletal muscles have been also implicated. Interestingly, the interaction between titin and sHSPs is enhanced by the expression of the N2B titin isoform. Consistently, we found strong co-localization between sHSPs and the sarcomeric cross striation pattern at more advanced ages when N2B titin isoform expression was high. In neonatal cardiomyocytes the more compliant N2BA is the dominant isoform, and sHSPs might not bind efficiently to compliant titin. Accordingly, we could not find prominent co-localization between sHSPs and titin in neonatal cardiomyocytes, thereby hypothetically leaving more functional groups of titin molecules accessible for oxidative insults.

Our present findings extend the above observations, and suggest that the increase in sHSPs expression can gradually outweight the increase in titin expression during cardiomyocyte development, thereby increasing the probability for interactions between sHSPs and titin isoforms. Moreover, our data did not support major distinctions between the oxidative characteristics of N2BA and N2B titin isoforms. Based on remarkably pronounced correlations

between the expression levels of HSP27 and αB-crystallin and oxidative changes in mechanical characteristics of cardiomyocytes (i.e. in $F_{passive}$) and biochemical (i.e. SH oxidation or carbonylation) properties of titin isoforms, we postulate a link between the oxidative sensitivities of titin isoforms and the expression levels of HSP27 and αB-crystallin.

In summary, this work implicated relationships between titin isoform oxidation, cardiomycyte $F_{passive}$ and HSP27/αB-crystallin expression in the developing rat heart. Two different types of oxidative modifications: SH-group oxidation and carbonylation of titin isoforms influenced $F_{passive}$ of cardiomyocytes in a similar fashion suggesting common molecular mechanisms for their coordinated effects. Our data suggest that during cardiac development the oxidative sensitivity of titin-based $F_{passive}$ declines due to the build-up of a protective mechanism preventing the oxidation of titin. We propose that this protection is mediated (at least in part) by sHSPs, such as HSP27 and αB-crystallin.

Increased compensatory Ca^{2+} -sensitivity in early left ventricular hypertrophy

In the second part of our study, we investigated the functional changes and myofilament system of early/compensated and advanced/decompensated stages of pathological myocardial hypertrophy in pressure overload-induced left ventricular hypertrophy rat model. We observed enhanced Ca^{2+}-sensitivity (pCa_{50}) and unaltered F_{max} of permeabilized left ventricular cardiomyocytes. It could be hypothesized that hyperphosphorylation of PKC-specific Thr-144 site of cTnI might have predominantly contributed to the increased myofilament Ca^{2+}-sensitivity in early stage of pathological left ventricular hypertrophy. Accordingly, the initially increased sarcomere Ca^{2+}-sensitivity regresses during the transition from compensated left ventricular hypertrophy to early-stage heart failure and it substantially reduces at heart failure.

The pressure overload -induced pathological left ventricular myocardial hypertrophy leads to heart failure. Distinct alterations could be observed left ventricular contractility in the early and late states of left ventricular hypertrophy. Based on the *in vivo* pressure-volume analysis

observation, the ventricular contractility markedly increased thus compensating for the post-load in the system, thus at this stage, the systolic function of the heart is preserved. However, the ventricle is only able to temporarily adapt to the changed conditions, the contractility, thus the systolic function reduced as a result of prolonged pressure overloading. The increased pressure overload affects the contractility of the ventricle at both the organ and sarcomere function levels.

In vivo operation and characterization of rat models of pathological hypertrophy were performed by our collaboration partners at the Semmelweis University Heart and Vascular Center. Increased pressure overload was provided by abdominal aortic banding operation for 6, 12 and 18 weeks, resulting in the development of significant left ventricular hypertrophy. Consistently with their previous experiences with this model and the *in vivo* haemodinamics measurements confirmed that early left ventricular hypertrophy developed at 6 weeks of aortic banding, characterized by impaired diastolic function and preserved systolic function. All this confirmed that the structural and molecular alterations were already present after 6 weeks of aortic banding.

Increased left ventricular contractility was observed in the early stage of myocardial hypertrophy, we observed enhanced Ca^{2+}-sensitivity (pCa_{50}) and unaltered F_{max} of cardiomyocytes.

Therefore, our results indicate that increased myofilament Ca^{2+}-sensitivity held responsibility for the contractility augmentation on the cellular level during the early-phase of pressure overload-induced left ventricular hypertrophy. Although, this kind of relation between myofilament Ca^{2+}-sensitivity and left ventricular contractility in pathological has not been postulated yet, numerous groups have reported increased sarcomere Ca^{2+}-sensitivity in pathological left ventricular hypertrophy with preserved systolic function. Furthermore, to define the underlying molecular mechanism for the increased myofilament Ca^{2+}-sensitivity, we

analyzed the phosphorylation status of specific regulatory sites of cTnI (Ser-23/24, Ser-43and Thr-144) and cMyBP-C (Ser-282). These measurements revealed a robust increment in the phosphorylation of the PKC-dependent Thr-144site of cTnI in the AB-6 weeks group. In line with our results, it has been previously reported that pseudo-phosphorylation of cTnI at Thr-143/144 (Thr-143 in human and Thr-144 in mouse/rat) potentially enhances myofilament Ca^{2+}-sensitivity. Interestingly, the hyperphosphorylation of Thr-143 site did not affect the maximal force development in human permeabilized cardiomyocytes, which finding is also in good agreement with our results demonstrating unaltered F_{max} in the AB-6 weeks group. Considering the fact that no alterations occurred at other sites of cTnI (Ser-22/23 and Ser-43) and cMyBP-C (Ser-282), it could be hypothesized that hyperphosphorylation of PKC-specific Thr-144 site of cTnI might have predominantly contributed to the increased myofilament Ca^{2+}-sensitivity in the AB-6 weeks group.

Our collaborators provided chronic pressure loading for 12 and 18 weeks in male rats to study the advanced stage of left ventricular hypertrophy, where increased left ventricular contractility regressed in the experimental groups. Consists with in vivo heamodinamics measurements, in parallel, we found that the cellular (enhancement of myofilament Ca^{2+}-sensitivity) and molecular (cTnI hyperphosphorylation at PKC-specific Thr-144 site) adaptations also diminished in the AB groups at 12 and 18 weeks.

The $F_{passive}$ of the isolated cardiomyocytes was not affected at all by diastolic functional impairment in the early (prolonged active relaxation) and advanced stages (active relaxation and passive tension) of left ventricular hypertrophy. In the stages of left ventricular hypertrophy we examined, therefore, it is unlikely that the passive elements of the sarcomere would play a primary role in myocardial stiffness. It seems, the passive elements of the sarcomere are not play primary role in the miocardium stiffness in this rat model.

Based on our findings and the previous literature data, we hypothesize that increment in myofilament Ca^{2+}-sensitivity (due to hyperphosphorylation of the PKC-specific Thr-144 site of cTnI) represents an early but only temporary adaptation to increased pressure overload. Accordingly, the initially increased sarcomere Ca^{2+}-sensitivity regresses during the transition from compensated left ventricular hypertrophy to early-stage heart failure and it substantially reduces at heart failure.

Original observations of the doctoral thesis

Based on the results of the doctoral thesis the following original statements are made:

- Oxidative sensitivity of passive tension gradually declines with cardiomyocytes age.
- The susceptibilities of N2BA and N2B titin isoforms for oxidative modifications are similar at a given cardiomyocyte age.
- The expression levels of myofibrillum associated sHSPs increase gradually in the developing heart and may confer protection against oxidative titin modifications.

- In a rat model of early pathological myocardial hypertrophy, hyperphosphorylation of PKC-mediated cardiac troponin I (Thr-144) is associated with increased Ca^{2+} -sensitivity of cardiomyocytes, which may be a compensatory response of the myofilament system to increased left ventricular pressure.

6. Summary

Diastolic stiffness of cardiomyocytes is regulated by both indirect (e.g., via heat shock proteins) and direct (e.g., oxidation effects) modifications of the giant titin protein which are very important in the physiological and pathological adaptation of the heart. According to data provided in this study, we conclude that the effect of oxidative posttranslational modifications (SH oxidation, carbonylation) of titin on passive stiffness gradually decrease during postnatal adaptation of the heart, which is due to the upregulation of titin-associated heat shock protein (HSP27 and αB-crystallin) expression after birth. Our data implicate a gradual build-up of a protective mechanism against titin oxidation during the maturation of the sarcomere structure. During various phases of the development of pressure overload-induced pathological left ventricular myocardial hypertrophy distinct alterations could be observed in left ventricular contractility and myofilament function contributing to the progression of heart failure. Hyperphosphorylation of PKC-mediated cardiac troponin I at Thr-144 underlies the increased Ca^{2+} -sensitivity of force production in permeabilized cardiomyocytes in the early stage of pressure overload-induced left ventricular hypertrophy. Data provided here implicate a compensatory response of the myofilament system for increased pressure loading in the left ventricle.

List of publications

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List of publications related to the dissertation

1. Ruppert, M., **Bódi, B.**, Korkmaz-Icöz, S., Loganathan, S., Jiang, W., Lehmann, L., Oláh, A., Barta, B. A., Sayour, A. A., Merkely, B., Karck, M., Papp, Z., Szabó, G. T., Radovits, T.: Myofilament Ca2+ sensitivity correlates with left ventricular contractility during the progression of pressure overload-induced left ventricular myocardial hypertrophy in rats.
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