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

Effects of protein carbonylation on the function of the myofilaments and its role in the postinfarction remodeling

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Head of the **Examination Committee**:	Prof. Dr. György Balla, MHAS
Members of the Examination Committee:	Dr. Péter Bay, PhD
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The Examination takes place at the Department of Pediatrics, Faculty of Medicine, University of Debrecen.
April 29, 2014. 11:00

Head of the **Defense Committee**:	Prof. Dr. György Balla, MHAS
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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen.
April 29, 2014. 13:00

1. Introduction and background

1.2. Myocardial infarction and postinfarction remodeling

Ischemic heart disease is worldwide one of the leading causes of death. Myocardial infarction (MI) is the myocardial necrosis caused by long-lasting ischemia mainly as a consequence of atherosclerotic plaque rupture and thrombotic coronary artery occlusion in the background.

During the past years, the mortality of the MI has been decreasing, thereby more and more patients survive the acute event, who have a great chance to develop heart failure as a consequence of the postinfarction myocardial remodeling. Chamber dilatation, left ventricular (LV) hypertrophy and failing LV systolic function are the clinical manifestations of the postinfarction remodeling, however the specified cellular and molecular mechanisms has not yet been clarified.

During the acute phase of the MI, the cardiomyocytes die, thereafter scar formation starts due to the limited endogenous regenerative capacity of the myocardium. As a consequence of the myocardial muscle loss, neurohumoral systems [e.g. sympathetic nervous system, renin-angiotensin-aldosteron system (RAAS)] are activated in the acute phase as a compensatory mechanism, however later they reach abnormal levels and contribute to the pathologic remodeling of the LV myocardium.

Moreover, the necrosis induces an intensive inflammatory response in the myocardium, which on one hand is necessary in the cleaning of the dead cellular components and in the scar formation, however on the other hand the released reactive species can further damage the surrounding structures.

These mechanisms have global effects on the left ventricle and the whole heart determining the outcome of the MI and the postinfarction remodeling.

The consequence of the above events are the changes in the cardiomyocyte structure and function, however the exact molecular and cellular mechanisms

has not been understood completely. One part of this work concentrates on the details of the postinfarction cardiomyocyte remodeling.

1.2. Organization of the contractile system and the mechanism of contraction

The ventricular myocardium is made up of numerous cell types, the most important are the cardiomyocytes, which are responsible for the myocardial contraction and thereby for the maintenance of the blood circulation.

The feature of these cells is the cross-striation pattern visible under the light microscope due to the organization of the contractile elements, the myofibrils. The thick and the thin contractile filaments consist of various contractile proteins [actin, myosin light chain-1 and -2 (MLC-1 and MLC-2), myosin heavy chains (MHC), troponin C, I, T (TnC, TnI, TnT), tropomyosin, myosin binding protein-C (MyBPC), alpha-actinin, desmin, etc.].

The basis of the contraction is the Ca^{2+} released in the cytoplasm of the cardiomyocytes which binds to the TnC leading to the cyclic interaction of the actin and the myosin, finally resulting in force generation. Ca^{2+}-dependency of the contractile force is often studied in „skinned", permeabilized cardiomyocyte preparations during isometric Ca^{2+}-contractures. Plotting the force values as a function of the $[Ca^{2+}]$ results in a sigmoid curve, called Ca^{2+}-force relationship or Ca^{2+} sensitivity curve. This curve gives information about the interfilament cooperativity and the Ca^{2+} sensitivity of force production. This latter parameter depends on the sarcomere length, the length-dependent Ca^{2+}-sensitisation is the molecular basis of the Frank-Starling mechanism. Moreover, various other factors and states (e.g. oxidation, phosphorylation) modulate the Ca^{2+} sensitivity of force production. This work focused on the postinfarction alterations and the effects of the oxidative stress.

1.3. Postinfarction remodeling of the contractile machinery

Numerous studies investigated the role of myofilament alterations in the postinfarction remodeling. The Ca^{2+} sensitivity of force production is one of the most studied contractile parameters, however contradictory data are available about the changes of this parameter as well as about the direction of the change during the remodeling. The modification of the Ca^{2+} sensitivity of force production is mainly a result of the posttranslational myofilament protein alterations. Postinfarction activation of the neurohumoral systems (RAAS and the sympathetic nervous system) - aiming the maintenance of the cardiac output – modifies the phosphorylation status of the contractile proteins [e.g.: protein kinase A (PKA)-mediated hypophosphorylation or protein kinase C (PKC)-dependent hyperphosphorylation]. Moreover, inflammatory processes, e.g. the oxidative myofilament protein alterations, may complement the above signaling pathways and can be responsible partly for the postinfarction myocardial dysfunction.

Myocardial ischemia develops predominantly in myocardial regions directly supplied by the occluded coronary arteries, yet MI-related LV remodeling may also involve the remote regions. However, it is not entirely clear whether neurohumoral and oxidative signals converge with identical effects in the ischemic and nonischemic myocardium following MI. Moreover, it is still obscure whether the contractile function of the surviving cardiomyocytes in the directly affected areas differs from those in the remote ones.

1.4. Reactive species and oxidative stress

Oxidative stress reflects an imbalance between antioxidant mechanisms and reactive oxygen/nitrogen species (ROS/RNS) produced by our cellular mechanisms, which plays a role in the pathogenesis of different cardiovascular diseases. The main sources of the reactive species in the cardiovascular system

are the mitochondria, however NADPH oxidases, xantin-oxidases, nitrogen oxide synthase, myeloperoxidase and lipoxigenases produce also ROS/RNS.

Under physiological conditions, the antioxidant mechanisms compensate for the detrimental effects of ROS/RNS, however oxidative stress is characterized by the excess of ROS/RNS. ROS are produced during the incomplete reduction of the oxygen, these species include the superoxide anion ($O_2^{\bullet-}$), the hydrogen-peroxide (H_2O_2), which is transformed with the contribution of metal ions to the hydroxil radicals (OH•) in the Fenton reaction.

These radicals produced under physiological conditions modulate the function of various proteins and signaling pathways thereby mediating vasoregulation, cell growth, programmed cell death, the ischemic preconditioning and they are crucial factors responsible for the immune response.

During oxidative stress predominant reactive species can damage all the cellular components, like nucleic acids, lipids and proteins. In the cardiovascular system, direct or indirect structural or functional modulation of the contractile proteins can lead to systolic or diastolic dysfunction.

1.5. Protein carbonylation

As a result of oxidation different protein modifications occur, like tyrosin nitration, sulfhydril (SH) oxidation, glutationylation or carbonylation. Protein carbonylation, one of the most commonly used biomarkers of the severe oxidative stress, is an irreversible oxidative modification that develops when reactive aldehydes or ketones are added to amino acid residues. Primary protein carbonylation occurs in metal-catalyzed oxidation on proline, arginine, lysine and threonine residues, induced by the production of hydroxyl radicals through the participation of H_2O_2 and iron(II) in the Fenton reaction. The Fenton reaction is widely employed to produce hydroxyl radicals and induce protein carbonylation during *in vitro* experiments. Protein carbonylation can modify the function of the affected proteins, it can induce protein degradation

or can modulate signaling pathways, as well. It plays a central role in the pathogenesis of numerous disorders, e.g. MI, ischemia/reperfusion injury, cardiac surgery procedures, diabetic cardiomyopathy or heart failure. However, few data are available as concerns how myofilament carbonylation modulates the contractile function of a single cardiomyocyte in the heart.

2. Aims

The main aims of our research were:

1. To investigate the functional effects of myofilament protein carbonylation on the contractile function of the human permeabilized cardiomyocytes.
2. To explore the molecular and cellular components of postinfarction myocardial remodeling in a murine model of myocardial infarction by examining separately the directly affected and the non-infarcted remote LV segments.
3. To study the role of the protein carbonylation in the myocardial postinfarction remodeling.

3. Methods

3.1. Tissue samples

3.1.1. Human left ventricular tissue samples

Human donor hearts derived from four general organ-donor patients (41- and 46-year-old women, and 53- and 57-year-old men). The cause of death was cerebral aneurysm rupture or haemorrhagic stroke. The donors did not reveal any sign of cardiac abnormalities and had not received any medication apart from short-term dopamine, noradrenaline and mannisol. The samples were stored at -80 °C. The experiments complied with the Helsinki Declaration of the World Medical Association and approved by the Hungarian Ministry of Health.

3.1.2. Murine model of myocardial infarction

The myocardial infarction model was developed in a partner laboratory in Vienna. In 16-week-old mice (n=25), MI was induced by the ligation of the left anterior descending coronary artery (LAD). Non-infarcted hearts from sham-operated animals served as controls (n=5). Ten weeks after surgery, transthoracic echocardiography was performed, and ejection fraction was calculated to characterize the LV systolic function. After beating hearts were excised, they were either fixed in formaldehyde and infarct size was determined in Masson's trichrome-stained LV sections, or they were dissected for later mechanical cardiomyocyte measurements or for biochemical assays: the infarcted area was separated from the non-infarcted, remote area of the left ventricle. The samples were stored at -80 °C.

3.2. In vitro protein carbonylation by the Fenton reaction

In vitro protein carbonylation was induced the same way both in human and murine LV samples. Isolated, permeabilized cardiomyocytes were incubated in a Fenton reaction mixture (consisting of $FeSO_4$, H_2O_2, ascorbic acid in relaxing solution) for 7 min at room temperature, either in the mechanical set-up or before oxyblot assays in test-tubes. A subset of the samples was treated with 10 mM dithithreitol (DTT) to reduce any oxidized SH groups. Further combined treatments were carried out during mechanical measurements.

3.3. Determination of myofilament protein carbonylation by oxyblot assay

The carbonyl group content of the myofilament proteins was determined with an oxyblot protein oxidation detection kit. LV tissue samples were dissolved to prepare protein homogenates and the carbonyl groups were derivatized to 2,4-dinitrophenylhydrazone. After gel electrophoresis, the proteins were transferred to nitrocellulose membranes, and probed by anti-

dinitrophenylhydrazone antibodies. Actin-, α-actinin- and MHC-specific antibodies were employed to estimate the total amounts of these proteins. Relative protein carbonylation values were expressed as carbonylation indices (CI), after normalization for protein amounts, where the CI of the control samples was taken as 1.

3.4. Determination of myofilament protein SH group oxidation

3.4.1. Ellman's reaction

SH content was evaluated by the Ellman's reaction in the human tissue samples. Permeabilized cardiomyocytes were treated at room temperature with Fenton solutions (containing H_2O_2 in the concentration range of $0\text{-}10^5$ μM). Samples treated with 2,2'-dithiodipyridine (DTDP) or DTT served as positive or negative controls. SH content was determined by incubation with the SH-sensitive Ellman's reagent which binding to the SH group results in a yellow product. The absorbance of the solutions was measured and was considered to be proportional to the SH content. The SH content was determined by means of calibration curves of N-acetyl-L-cysteine as standard and the SH contents of the myocardial samples were calculated for 1 mg protein.

3.4.2. SH-specific biotinilation and Western immunoblot

Due to the small amount of the myocardial samples, SH oxidation was investigated by biotinilation in the murine model. Protein homogenates were divided into two parts. The first part was treated with DTT in order to reduce the disulfide bridges to SH groups, while the non-treated second part was used to compare the SH contents of the different myocardial tissue samples. After biotinilation, gel electrophoresis and blotting, a streptavidin-peroxidase system was used for SH signal detection. Anti-actin antibody was used to determine the total amount of actin. Relative SH oxidation was calculated by

normalization for the amount of actin, where the relative SH oxidation of the DTT-treated samples were regarded as 100%.

3.5. Investigation of the phosphorylation status of cardiac TnI

Protein lysates were boiled in 2x SDS sample buffer, and after gel electrophoresis and blotting, TnI phosphorylation-sensitive (S22/23 or T143) and insensitive antibodies were used to determine the levels of TnI phosphorylation and the amount of TnI, respectively. Chemiluminescence method (ECL) and autoradiography was used to visualize the signals. Relative phosphorylation was calculated after a normalization step. The level of TnI phosphorylation of the control samples was regarded as 100%.

3.6. Carbonylation of recombinant troponin complexes

Recombinant troponin complexes (Tn) containing TnT, TnC and dephosphorylated (TnI) or phosphorylated TnI (TnI-P) were exposed to Fenton treatment (as above) to investigate whether TnI phosphorylation affected protein carbonylation. TnI phosphorylation status was proven by phosphorylation-specific antibodies. Carbonylation was tested by oxyblot assay, protein amounts were determined by Sypro ruby blot staining. After normalization, carbonylation indices were compared.

3.7. Isometric force measurements in permeabilized cardiomyocyte preparations

The contractile function of both the human and murine cardiomyocytes was tested during isometric force measurements. Using frozen LV tissue samples, after mechanical isolation, permeabilization was performed to ensure the controlled intracellular circumstances for the measurements of the contractile functions. Single cardiomyocytes were mounted to the mechanical force measurement system. Repeated activation–relaxation cycles were performed at 15 °C at a sarcomere length (SL) of 1.9 μm or 2.3 μm to evoke Ca^{2+}-

contractures. Ca^{2+}-concentrations were expressed in negative logarithmic units as pCa values ($-\log_{10}[Ca^{2+}]$=pCa). During the Ca^{2+}-contractures the total force (F_{total}) and the passive force ($F_{passive}$) could be measured, while the active force (F_{active}) could be calculated and the actin-myosin cycling rate ($k_{tr,max}$) could be estimated. Moreover, information could be gained about the Ca^{2+} sensitivity of force production (pCa_{50}) from the Ca^{2+}-force relationship.

3.7.1. Investigation of the functional effects of the posttranslational protein modifications

The effect of protein phosphorylation was investigated by *in vitro* PKA treatments, SH-reagent DTT treatment was used to test the reversibility and the effects of the SH oxidation, while Fenton reagents were used to study the effects of protein carbonylation. To investigate the possible interaction between protein carbonylation and phosphorylation, combined treatments were carried out on further cardiomyocytes by application of the Fenton treatment before and after PKA exposure, or after incubation with protein phosphatase-1 (PP1c) or -2A (PP2Ac). Ca^{2+}–force relationships were determined before and after the treatments at a SL of 2.3 μm.

3.8. Data analysis, statistics

The Ca^{2+}–force relations were fitted to a modified Hill equation, while the force redevelopment after the release–restretch manoeuvre was fitted to a single exponential function. Each experimental preparation was fitted individually, the fitted parameters were pooled, and the mean values are reported. Statistical analyses were performed with GraphPad Prism 5.02 software. The numerical data are given as mean values ± SEM. Student's t-tests or analysis of variance (ANOVA) followed by the Bonferroni post hoc test were used to compare the experimental groups. Linear regression analysis was used to test the possible correlation between the changes in the Ca^{2+}

sensitivity (ΔpCa_{50}) and the degree of protein carbonylation. P values <0.05 were considered statistically significant.

4. Results

4.1. Myofilament protein carbonylation in human cardiomyocytes

Fenton chemistry was used to induce protein carbonylation in human cardiomyocytes as an *in vitro* model. Fenton treatment with increasing H_2O_2 concentrations increased carbonyl group formation in the proteins of the human permeabilized cardiomyocytes. The affected myofilament proteins were identified on the basis of their molecular masses: MLC-1 (20 kDa), actin (43 kDa), desmin (55 kDa), α-actinin (100 kDa), MyBPC (140 kDa) and MHC (200 kDa). The mean levels of carbonylation for all myocardial proteins suggested a sigmoidal H_2O_2 concentration dependence (in the Fenton mixture) with an apparent saturation around 10^5 μM H_2O_2 (Control: 1.00±0.05; 0 μM H_2O_2: 1.05±0.06; 10^0 μM H_2O_2: 1.08±0.06; 10^2 μM H_2O_2: 1.27±0.07; 10^3 μM H_2O_2: 1.86±0.11; $3*10^3$ μM H_2O_2: 3.38±0.32*; $3*10^4$ μM H_2O_2: 6.22±0.75*; 10^5 μM H_2O_2: 6.80±0.82*; in CI units; P<0.05). The relative protein carbonylation appeared to be higher than the average for the proteins at the molecular masses of MLC-1, MyBPC, and α-actinin, whereas it was less for the proteins at the molecular masses of desmin, actin and MHC.

Ellman's assay was utilized to investigate the Fenton reaction-induced SH group oxidation of the myocardial proteins. Fenton treatment displayed an SH group oxidizing effect (from the control of 471.6±59.1 μM/mg protein to 201.6±21.4 μM/mg protein, P<0.05), which was significant at the highest applied H_2O_2 concentration (Fenton 10^5), but not at the lower concentrations. DTDP treatment - used as a positive control - decreased the SH group content even further (to 83.1±15.3 μM/mg protein; P<0.05 vs. Control), whereas the SH group oxidation was reversed by the SH-reagent DTT treatment both after

Fenton treatment and after DTDP application (599.3±102.1 μM/mg protein and 621.2±107.3 μM/mg protein, respectively; P<0.05 vs. before DTT). Hence, the level of SH groups was a function of the Fenton treatment, but only with the use of extreme high H_2O_2 concentrations.

To investigate the mechanical consequences of myofilament protein carbonylation, permeabilized human cardiomyocytes (n=6) were treated with Fenton solution in a mechanical force measurement set-up similarly as in the biochemical assays. The concentration-dependent effects of the Fenton reaction on the F_{active} were first obtained. No major structural changes were seen at any time throughout the experiments. Nevertheless, a gradual decline in F_{active} was observed in parallel with increasing oxidative stress [i.e. with increasing concentrations of H_2O_2, in the presence of constant iron(II) and ascorbic acid concentrations (Control: 100.0%; Fenton 0: 98.9±3.3%; Fenton 10^0: 95.0±3.9%; Fenton 10^2: 89.1±3.7%*; Fenton 10^3: 68.3±4.1%*; Fenton $3*10^3$: 54.9±4.7%*; Fenton 10^4: 36.0±3.7%*; Fenton $3*10^4$: 3.5±1.6%*; Fenton 10^5: 0.3±0.1%*; in relative units; P<0.05 vs. Control). This decrease in F_{active} reflected the increase in the CI for myocardial proteins in this H_2O_2 concentration range. A group of isolated human cardiomyocytes (n=6) was subjected to a single exposure to Fenton solution containing $3*10^3$ μM H_2O_2 to test its effects on the Ca^{2+} sensitivity of force production (pCa_{50}), F_{active} and $F_{passive}$. Subsequent DTT exposures were used to test the reversibility, and to estimate the SH-sensitive components of the observed mechanical changes. A clear reduction in the Ca^{2+} sensitivity of force production was observed following incubation in the presence of this Fenton solution [pCa_{50}(Control): 5.74±0.01; pCa_{50}(Fenton): 5.65±0.01; P<0.05]. Besides the decreased Ca^{2+} sensitivity of force production, a reduced F_{active} and an increased $F_{passive}$ were detected [F_{active}(Control): 11.39±1.63 kN/m^2; F_{active}(Fenton): 8.66±1.06 kN/m^2*; $F_{passive}$(Control): 1.72±0.21 kN/m^2; $F_{passive}$(Fenton): 2.33±0.22

kN/m^2*; P<0.05]. The rate constant of tension redevelopment ($k_{tr,max}$) and the Hill coefficient (n_{Hill}) were not affected by the *in vitro* induced carbonylation [$k_{tr,max}$(Control): 0.68±0.06 1/sec; $k_{tr,max}$(Fenton): 0.57±0.02 1/sec; n_{Hill}(Control): 2.42±0.05; n_{Hill}(Fenton): 2.31±0.06]. Subsequent DTT treatment affected none of these parameters [pCa_{50}(DTT): 5.63±0.01; F_{active}(DTT): 7.14±1.10 kN/m^2; $F_{passive}$(DTT): 2.44±0.28 kN/m^2; $k_{tr,max}$(DTT): 0.55±0.07 1/sec; n_{Hill}(DTT): 2.43±0.10; P>0.05 vs. Fenton; P<0.05 vs. Control]

4.2. Postinfarction remodeling in murine cardiomyocytes

Ten weeks after surgery, transthoracic echocardiography was performed on the mice in the partner laboratory in Vienna. LV systolic function was significantly reduced as compared to the controls. Afterwards in Masson's trichrome-stained sections the size of the infarct was measured, and 50.3±5.7% of the left ventricle was involved by the infarction.

Isometric force measurements were performed in permeabilized LV cardiomyocytes isolated from both experimental groups and measured their contractile parameters. The remaining cardiomyocytes in the infarcted area had a lower Ca^{2+} sensitivity of force production than that of those in the remote non-infarcted area or that of those in the control left ventricles at both shorter and longer SLs [pCa_{50}; Control(1.9 μm): 5.81±0.02; MI inferior(1.9 μm): 5.80±0.02; MI anterior(1.9 μm): 5.73±0.03*; Control(2.3 μm): 5.91±0.02; MI inferior(2.3 μm): 5.88±0.02; MI anterior(2.3 μm): 5.81±0.03*; P<0.05 vs. Control at the same SL]. However, the length-dependent Ca^{2+} characteristics of the contractile system were approximately the same in all groups. Moreover, no differences were found in the mean F_{active}, $F_{passive}$ and $k_{tr,\max}$ or in the visual appearances of the cardiomyocytes from the various myocardial regions.

To elucidate the molecular background of the decreased Ca^{2+} sensitivity of isometric force production in the cardiomyocytes from the anterior wall, first, the level of specific TnI phosphorylation was investigated. The extent of

phosphorylation at the PKC-specific threonine 143 site did not differ significantly among the three groups. However, the PKA-specific serine 22 site of TnI was less extensively phosphorylated in the cardiomyocytes in the infarcted anterior area than in the controls. In line with these results, saturation of these PKA-specific phosphorylation sites by *in vitro* incubation in the presence of PKA enzyme resulted in a small, but significant decrease in pCa_{50} in the MI anterior cardiomyocytes, but not in the control ones.

We next set out to assess the impact of putative oxidative protein modifications on the Ca^{2+} sensitivity of force production. The relative amount of reduced SH groups of actin was significantly lower in the MI anterior region than that in the control group, but not in the remote inferior myocardium, suggesting a higher degree of SH oxidation in the infarcted anterior myocardium (Control: 83.8±6.2%; MI inferior: 62.5±10.3%; MI anterior: 49.1±10.6%*; P<0.05). *In vitro* DTT treatment of the protein homogenates increased the reduced SH group content in the homogenates of MI hearts (100.0%), and hence indicated the reversibility of this oxidative modification. Nevertheless, *in vitro* DTT treatment did not restore the decreased Ca^{2+} sensitivity of force production of MI anterior cardiomyocytes [pCa_{50}(before DTT): 5.79±0.02; pCa_{50}(after DTT): 5.76±0.02; P>0.05].

Oxyblot assays were performed in cardiomyocyte protein homogenates from control and infarcted hearts to investigate the levels of protein carbonylation. The degrees of actin (Control: 1.00±0.04; MI inferior: 1.40±0.12; MI anterior: 1.46±0.18*; P<0.05 vs. Control) and MHC carbonylation (Control: 1.00±0.07; MI inferior: 1.46±0.17; MI anterior: 2.06±0.46*; P<0.05 vs. Control) were significantly higher at the MI anterior wall than those in the control left ventricles. In the case of α-actinin (Control: 1.00±0.07; MI inferior: 0.95±0.09; MI anterior: 1.25±0.16; P>0.05) and MyBPC (Control: 1.00±0.14; MI inferior: 0.82±0.11; MI anterior: 0.70±0.14; P>0.05) similar CIs were detected.

To verify the hypothetical link between myofilament protein carbonylation and the decreased Ca^{2+} sensitivity of force production, an *in vitro* Fenton reaction was employed. Intense protein carbonylation and a limited degree of SH oxidation (relative SH content; control: 100.0±1.4%; Fenton: 77.8±3.5%) were observed in the control cardiomyocytes. The potentially complicating effects of SH oxidation were minimized by sequential DTT treatment. Similarly to the results in the infarcted hearts, comparable degree of enhanced protein carbonylation of actin and MHC was observed in permeabilized control cardiomyocytes following exposure to the Fenton reagents. Protein carbonylation was not affected by *in vitro* DTT treatment. The application of Fenton solution in isolated control cardiomyocytes resulted in a significant decrease in the Ca^{2+} sensitivity of force production [pCa_{50}(Control): 5.76±0.03; pCa_{50}(Fenton): 5.62±0.04; $P<0.05$] which was not affected by DTT treatment. Finally, an apparent linear relationship was seen when the differences between the control pCa_{50} values and those from the MI anterior area, the MI inferior area and Fenton-treated cardiomyocytes (ΔpCa_{50}) were expressed as a function of the relative protein carbonylation of actin ($P=0.0264$; $r^2=0.8661$) or MHC ($P=0.0114$; $r^2=0.9117$). To test the possible interactions between protein phosphorylation and carbonylation, the effects of Fenton reagents on the Ca^{2+} sensitivity of force production were tested in different phosphorylation stages: before or after PKA treatments or after protein phosphatase treatments (PP1c or PP2Ac). In accordance with previous results, PKA exposures had no significant effect on the Ca^{2+} sensitivity in control cardiomyocytes either before or after Fenton treatment, while PP1c decreased, whereas PP2Ac increased the Ca^{2+} sensitivity of force production. The degree of the decrease in the Ca^{2+} sensitivity of force production induced by the Fenton reaction was independent of the phosphatase and kinase treatments. Our results revealed that the functional effect of the Fenton

treatment on the Ca^{2+} sensitivity of force production is independent of the phosphorylation status of myofilament proteins. This hypothesis was proven when recombinant Tn complexes were assayed for protein carbonylations by *in vitro* Fenton treatment, where TnI phosphorylation did not appear to modulate carbonylations of the subunits of the Tn complex.

5. Discussion

5.1. Contractile protein carbonylation modulates the human cardiomyocyte contractility

The first part of the study investigated the direct effects of protein carbonylation on the contractile function of human cardiomyocytes. To this end, Fenton chemistry was used to induce protein carbonylation through hydroxyl radical production. The use of permeabilized cardiomyocytes allowed us the characterization of the relationship between the level of myofilament carbonylation and quantitative indices of isometric force production. Interestingly, the carbonylation seems to disturb the function and the fine regulation of cardiomyocyte contractility in the lack of any visible microscopic structural alterations.

Several different types of posttranslational oxidative contractile protein modifications (e.g. SH group oxidation, protein nitration, protein carbonylation, etc.) have been reported as possible mediators of contractile dysfunction during oxidative stress in the heart. The mechanical consequences have also been elucidated for some, but not all of the above protein changes, practically no information is currently available as concerns the mechanical effects of contractile protein carbonylation in human cardiomyocytes.

This oxidative modification has been reported in several cardiovascular diseases, like in right heart failure, in ischemia/reperfusion injury, after coronary microembolization or in postinfarction remodeling. These data show

that protein carbonylation is a significant oxidative marker which plays a role in various pathological processes, however the exact mechanism is not clear.

In the present study, the effects of contractile protein carbonylation were provoked *in vitro* by the Fenton reaction in human permeabilized cardiomyocytes. This reaction is observed in failing cardiac myocytes and in ischemia–reperfusion injury, and it is widely employed to produce hydroxyl radicals for *in vitro* experiments. The advantage of the combination of the Fenton reaction with the study of isolated, permeabilized cardiomyocytes is that it excludes interference with membrane components, intracellular organelles and elements of the Ca^{2+} homeostasis.

Oxidative protein modifications other than protein carbonylation cannot be excluded for hydroxyl radical exposures. In our studies, we paid specific attention to SH oxidation, and significant levels of SH oxidation and functional effects could not be detected showing that the underlying molecular mechanism is not related to SH oxidation. On the other hand, the contractile proteins of the human cardiomyocytes proved to be highly sensitive to carbonylation. Exposure to the increasing concentrations of H_2O_2 in the Fenton reagent enhanced the level of protein carbonylation and reduced the maximal isometric force in a graded fashion. It is unlikely that the highest concentrations of radicals produced by this method are present under physiological conditions. Nonetheless, free radical concentrations might reach levels sufficiently high to provoke relevant levels of protein carbonylation *in vivo* in diffusion-limited compartments. In our *in vitro* study, hydroxyl radicals were generated in the direct vicinity of cardiomyocytes and modified a number of myofilament proteins and the cardiomyocyte contractility. However, it is uncertain whether the carbonylation of a selected single protein alone or that of a group of proteins was responsible for the observed mechanical changes.

Oxidative protein modifications modulating the contractile system may lead to systolic or diastolic dysfunction during conditions of oxidative stress. In our present experiments, protein carbonylation decreased active force production and its Ca^{2+} sensitivity. These changes may contribute to the development of weaker myocardial contractions and hence to a systolic dysfunction. We also report here that myofilament protein carbonylation increases the Ca^{2+}-independent passive force, thereby implicating a potential molecular mechanism for increased cardiomyocyte stiffness in diastolic heart failure.

Importantly, a reduced Ca^{2+} responsiveness and/or contractile dysfunction are frequently referred to in clinical conditions in association with oxidative stress. Among others, myocardial stunning is also characterized by excessive oxidative stress and reduced myofilament Ca^{2+} sensitivity, however the mechanism is not clear and the main target cellular component has not been clarified. The present results suggest that myofilament carbonylation may be regarded as a potential link between myofilament oxidation and the contractile dysfunction in the above conditions.

5.2. The role of myofilament carbonylation in the postinfarction remodeling of the contractile system

The second part of my work concentrated on region-specific characteristics of postinfarction LV remodeling 10 weeks after MI induced by LAD ligation in mice. Cardiomyocytes from the infarcted anterior and from the non-infarcted remote inferior LV region were studied separately. In the infarcted area, a marked decrease in the Ca^{2+} sensitivity of force production was observed, in parallel with pronounced oxidative myofilament protein changes (SH oxidation and carbonylation). Likewise, selective experimental protein carbonylation in the control cardiomyocytes provoked a decrease in the Ca^{2+} sensitivity of force production irrespective of the phosphorylation status of the myofilaments. Moreover, an apparent linear relationship was observed

between the extent of change in pCa_{50} and the level of protein carbonylation. Hence, we conclude that myofilament protein carbonylation has the potential to reduce the Ca^{2+} sensitivity of force production in the remaining cardiomyocytes in the infarcted area, and may contribute to the regional contractile dysfunction in hearts following MI.

Postinfarction remodeling and the consequent LV dysfunction is a hot field in the cardiovascular physiology. Several investigations have proven the significance of the changes in myofilament Ca^{2+} sensitivity in the systolic dysfunction and in the development of the postinfarction heart failure. Nevertheless, conflicting data are available about the direction and magnitude of the postinfarction changes of the Ca^{2+} sensitivity. The reason for this discrepancy is multifactorial (species, model and temporal characteristics), on the other hand these results can be parts of a mosaic which can help us to build-up the process of the postinfarction remodeling. The above studies focused primarily on non-infarcted areas of the left and/or right ventricles, whereas we investigated both the MI-affected and non-infarcted LV regions.

At 10 weeks after coronary artery ligation-induced myocardial infarction, surviving cardiomyocytes could be identified in the infarcted region of the left ventricle characterized by decreased Ca^{2+} sensitivity of force production.

The Ca^{2+} sensitivity of force production is closely connected with TnI and MyBPC phosphorylation under physiological conditions. Our results also confirm that TnI phosphorylation is strongly linked with the Ca^{2+} sensitivity of force production, however, in our present study neither PKA- nor PKC-dependent TnI phosphorylation appeared to be directly responsible for the decrease in the Ca^{2+} sensitivity of force production in cardiomyocytes from the infarcted region, and this suggested the involvement of additional molecular mechanisms. This led us to the oxidative protein modifications, mainly SH oxidation and protein carbonylation occured as possible factors resulting in

decreased Ca^{2+} sensitivity of force production. Our results suggest that the relatively small degree of SH oxidation in the MI anterior cardiomyocytes was not responsible for the decreased Ca^{2+} sensitivity of force production.

Protein carbonylation is widely accepted as a marker of severe oxidative stress, and it has been detected in several studies as a participant of the postinfarction remodeling. In the present study, increased levels of actin and MHC carbonylation were observed in cardiomyocytes from the infarcted region of murine hearts. To clarify the possible pathophysiological role of protein carbonylation, *in vitro* experiments were performed in control murine cardiomyocytes, in which actin and MHC carbonylation could also be provoked through hydroxyl radical formation. Moreover, a positive correlation was found between the decrease in the Ca^{2+} sensitivity and the degree of carbonylation. In parallel with previous studies, our results suggest that the different types of posttranslational myofilament alterations (phosphorylation, carbonylation) modify the contractile function, mainly the Ca^{2+} sensitivity of force production. The activity of the enzymes regulating the contractile functions could also be modified by the oxidative modifications. Therefore the possible interactions between protein phosphorylation and carbonylation were tested. After the application of PKA and phosphatase enzymes on permeabilized cardiomyocytes, we conclude that the mechanical effect of myofilament carbonylation seemed to be independent of the phosphorylation.

Overall, our data revealed that the protein carbonylation induced by experimental MI is associated with decreased myofilament Ca^{2+} sensitivity in the surviving cardiomyocytes of the infarcted zone. It additionally emerged that the effects of other mechanisms that regulate the Ca^{2+} sensitivity may be overridden by this protein modification. The reduction in Ca^{2+} sensitivity of force production renders the surviving myocardium in the infarcted zone hypocontractile, contributing to the global LV dysfunction.

6. Summary

The epidemiological burden of myocardial infarction and the postinfarction remodeling-related heart failure is growing nowadays. Exploration of the underlying molecular mechanisms may contribute to the better understanding of the patophysiology and thereby to the innovation of new therapeutical targets. Oxidative stress is one component of the acute event and the postinfarction remodeling resulting in oxidative posttranslational protein modifications.

During our experiments the direct effects of myofilament protein carbonylation on the contractile system were investigated in human myocardial tissue samples. On the other hand the role of protein carbonylation in the postinfarction remodeling was studied in a murine model of myocardial infarction.

During our investigations we made the following key observations: 1) Carbonylation of the contractile proteins in the isolated human cardiomyocytes could be induced by the *in vitro* Fenton reaction. 2) Myofilament carbonylation affects directly the function of contractile system, modifying the isometric active force, the passive stiffness of the cardiomyocytes and the Ca^{2+} sensitivity of force production. 3) Decreased Ca^{2+} sensitivity of force production was detected in the infarcted area of the left ventricle. 4) Protein carbonylation is a key factor in the determination of the decreased Ca^{2+} sensitivity of force production.

Our data indicate that myofilament protein carbonylation has direct effects on the function of the cardiomyocyte contractile system, it plays a central role in the left ventricular postinfarction remodeling and thereby contributes to the development of the postinfarction left ventricular dysfunction.

7. *In extenso* publications of the author

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Candidate: Ágnes Balogh
Neptun ID: I8HS03
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MTMT ID: 10036795

List of publications related to the dissertation

1. **Balogh, Á.**, Tóth, A., Pásztorné Tóth, E., Nagy, L., Kovács, Á., Kalász, J., Contreras, G.A., Édes, I., Papp, Z.: Myofilament carbonylation modulates contractility in human cardiomyocytes. *Exp. Clin. Cardiol. "accepted by publisher"*, 2014.
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List of other publications

3. Bodnár, D., Geyer, N., Ruzsnavszky, O., Oláh, T., Hegyi, B., Sztretye, M., Fodor, J., Dienes, B., **Balogh, Á.**, Papp, Z., Szabó, L., Müller, G., Csernoch, L., Szentesi, P.: Hypermuscular mice with mutation in the myostatin gene display altered calcium signaling. *J. Physiol.-London. "accepted by publisher"*, 2014.
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4. Vandenwijngaert, S., Pokreisz, P., Hermans, H., Gillijns, H., Pellens, M., Bax, N.A.M., Coppiello, G., Oosterlinck, W., **Balogh, Á.**, Papp, Z., Bouten, C.V.C., Bartunek, J., D'hooge, J., Luttun, A., Verbeken, E., Herregods, M.C., Herijgers, P., Bloch, K.D., Janssens, S.: Increased Cardiac Myocyte PDE5 Levels in Human and Murine Pressure Overload Hypertrophy Contribute to Adverse LV Remodeling.
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