Myofilament protein carbonylation contributes to the contractile dysfunction in the infarcted LV region of mouse hearts

Ágnes Balogh¹, David Santer², Enikő T. Pásztor¹, Attila Tóth¹, Dániel Czuriga¹, Bruno K. Podesser²,³ Karola Trescher²,³, Kornelia Jaquet⁴, Ferenc Erdődi⁵, István Édes¹, and Zoltán Papp¹*

¹Division of Clinical Physiology, Research Center for Molecular Medicine, Institute of Cardiology, Faculty of Medicine, Medical and Health Science Center, University of Debrecen, Móricz Zs. krt. 22, Debrecen H-4032, Hungary; ²Ludwig Boltzmann Cluster for Cardiovascular Research, Medical University of Vienna, Vienna, Austria; ³Department of Cardiac Surgery, LK St Pölten, St Pölten, Austria; ⁴Research Group Molecular Cardiology, Bergmannsheil and St Josef Hospital, Clinics of the Ruhr-University of Bochum, Bochum, Germany; and ⁵Department of Medical Chemistry, Faculty of Medicine and Health Science Center, University of Debrecen, Debrecen, Hungary

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Aims The region-specific mechanical function of left ventricular (LV) murine cardiomyocytes and the role of phosphorylation and oxidative modifications of myofilament proteins were investigated in the process of post-myocardial infarction (MI) remodelling 10 weeks after ligation of the left anterior descending (LAD) coronary artery.

Methods and results Permeabilized murine cardiomyocytes from the remaining anterior and a remote non-infarcted inferior LV area were compared with those of non-infarcted age-matched controls. Myofilament phosphorylation, sulphydryl (SH) oxidation, and carbonylation were also assayed. Ca²⁺ sensitivity of force production was significantly lower in the anterior wall (pCa50: 5.81 ± 0.03, means± SEM, at 2.3 μm sarcomere length) than that in the controls (pCa50: 5.91 ± 0.02) or in the MI inferior area (pCa50: 5.88 ± 0.02). The level of troponin I phosphorylation was lower and that of myofilament protein SH oxidation was higher in the anterior location relative to controls, but these changes did not explain the differences in Ca²⁺ sensitivities. On the other hand, significantly higher carbonylation levels, [e.g. in myosin heavy chain (MHC) and actin] were observed in the MI anterior wall [carbonylation index (CI), CI actin: 2.06 ± 0.46, CI MHC: 1.46 ± 0.18] than in the controls (CI: 1). In vitro Fenton-based myofilament carbonylation in the control cardiomyocytes also decreased the Ca²⁺ sensitivity of force production irrespective of the phosphorylation status of the myofilaments. Furthermore, the Ca²⁺ sensitivity correlated strongly with myofilament carbonylation levels in all investigated samples.

Conclusion Post-MI myocardial remodelling involves increased myofilament protein carbonylation and decreased Ca²⁺ sensitivity of force production, leading potentially to contractile dysfunction in the remaining cardiomyocytes of the infarcted area.

Keywords Contractile function • Infarction • Myocytes • Remodelling • Sarcomere

1. Introduction

A large number of cardiomyocytes die via apoptosis and necrosis during myocardial infarction (MI), and the remaining cardiomyocytes often enter a deleterious remodelling process leading to cardiac dysfunction. The functional cardiomyocyte changes involve the intracellular Ca²⁺ handling and Ca²⁺ sensitivity of force production of the contractile machinery in the remodelled heart. Previous publications have reported various, often conflicting post-MI-linked myofilamentary alterations. For example, the Ca²⁺ responsiveness of the contractile protein machinery was found to be either decreased, unaltered, or increased. Different molecular alterations, mostly post-translational myofilament protein modifications have been suggested for the explanation of the above mechanical abnormalities. In this context, prolonged neurohumoral activation with consequent maladaptive compensatory changes at the level of the cardiomyocytes (e.g. down-graded β-adrenergic signalling) may potentially lead to altered protein phosphorylation. A pathogenic role for protein kinase A (PKA)-dependent cardiac troponin I (TnI)
hypophosphorylation or increased protein kinase C (PKC)-mediated myofilament protein phosphorylation has been implicated during this process. Moreover, inflammatory processes, e.g., the participation of reactive oxygen and nitrogen species leading to oxidative myofilament protein alterations, may complement the above-mentioned signalling pathways. Indeed, Canton et al. reported on the oxidation of actin and tropomyosin in post-ischaemic rat hearts, and tropomyosin oxidation was demonstrated in porcine and canine myocardial preparations after coronary microembolizations. Interestingly, the level of tropomyosin oxidation correlated inversely with the contractile function in post-ischaemic porcine hearts, suggesting that oxidative myofilament alterations might be responsible, at least in part, for the post-ischaemic myocardial dysfunction.

Myocardial ischaemia develops predominantly in myocardial regions directly supplied by the occluded coronary arteries, yet MI-related left ventricular (LV) remodelling may also involve remote regions. However, it is not entirely clear whether neurohumoral and oxidative signals converge with identical effects in the ischaemic and non-ischaemic 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.

We have now investigated the region-specific characteristics of post-ischaemic myocardial remodelling in a mouse model of MI. Direct force measurements in permeabilized cardiomyocytes allowed a comparison of the post-MI remodelling in the infarcted anterior and non-infarcted inferior LV areas in murine hearts 10 weeks after ligation of the left anterior descending (LAD) coronary artery, i.e., long after the acute necrotic event. Cardiomyocyte force measurements and parallel biochemical assays allowed the recognition of myofibrillar protein modifications that are critical as concerns the post-ischaemic mechanical dysfunction.

Our results pointed to a hierarchical relationship among the effects of the post-MI remodelling-related myofilament protein alterations, and suggested that myofilament carbonylation may mask the mechanical effects of other post-translational changes in the remaining myocardium of the infarcted zone.

2. Methods

Detailed materials and methods are provided in Supplementary material online.

2.1 Animals

Female OF-1 mice (32.9 ± 0.5 g, 16 weeks old, n = 30) were anaesthetized with i.p. ketamine (100 mg/kg) and xylazine (12 mg/kg). After intubation, room air ventilation was provided (HSE Minivent; Harvard Apparatus, March-Hugstetten, Germany). After left thoracotomy, the pericardium was opened, the LAD was ligated (8–0 Prolene suture), and the chest was closed after de-airing (MI group, n = 25). Post-operatively, the mice received i.p. ketamine (100 mg/kg) and xylazine (12 mg/kg). After intubation, mechanical ventilation was provided (HSE Minivent; Harvard Apparatus, March-Hugstetten, Germany). After left thoracotomy, the pericardium was opened, the LAD was ligated (8–0 Prolene suture), and the chest was closed after de-airing (MI group, n = 25). Post-operatively, the mice received i.p. ketamine (100 mg/kg) and xylazine (12 mg/kg). After intubation, mechanical ventilation was provided (HSE Minivent; Harvard Apparatus, March-Hugstetten, Germany).

2.2 Echocardiography

Ten weeks after surgery, transthoracic echocardiography (Vevo 770; Visualsonics, Toronto, Canada; mouse cardiac scanhead RMV707B) was performed under sedation (100 mg/kg ketamine, 12 mg/kg xylazine, i.p.). Systolic and diastolic LV dimensions and ejection fraction (EF) were determined in the two-dimensional parasternal short-axis view of the LV at the mid-papillary muscle level.

2.3 Infarct size determination

Under anaesthesia (see above), beating hearts were excised and were either fixed in 4% formaldehyde for histological analyses, or 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. Samples were weighed and stored at −80 °C. The infarct size was determined in Masson’s trichrome-stained LV sections as described previously.

2.4 Force measurements in permeabilized cardiomyocyte preparations

Using frozen LV tissue samples, after mechanical isolation, permeabilization was performed with 0.5% Triton-X 100 detergent. The technique employed for force measurements in cardiomyocyte-sized preparations has been detailed earlier. Repeated activation—relaxation cycles were performed in single cardiomyocytes at 15 °C (to maintain the stability of the preparations), first at a sarcomere length (SL) of 1.9 μm and then at an SL of 2.3 μm. Isometric force values were normalized for the maximal Ca2+-activated active force, and Ca2+-force relationships were fitted to a modified Hill equation to determine the Ca2+ sensitivity of isometric force production, i.e., pCa50. Active isometric force (F0), Ca2+-independent passive force (F passive), and the rate tension of force redevelopment (kTR,max) were determined.

Additionally, the effect of PKA (catalytic subunit of bovine heart PKA; Sigma-Aldrich, St Louis, MO, USA) on pCa50 was also measured. To assess the mechanical effects of sulphydryl (SH) oxidation, antioxidant treatment (10 mM dithiothreitol (DTT)) was applied. Ca2+-force relationships were determined at an SL of 2.3 μm before and after in vitro PKA (40 min) or DTT treatment (30 min).

2.5 Investigation of the phosphorylation status of cardiac TnI

A modified radio-immunoprecipitation assay (RIPA) solution was used for the preparation of protein lysates. After PAGE and blotting, TnI phosphorylation-sensitive and -insensitive antibodies were used to determine the levels of TnI phosphorylation and the amount of TnI. Relative phosphorylation was calculated after a normalization step. The level of phosphorylation of the control TnI was regarded as 100%.

2.6 Detection of protein carbonyl groups

An oxyblot protein oxidation detection kit (Millipore, Billerica, MA, USA) was used to detect the carbonyl group content of myofilament proteins induced by oxidative stress. Myocardial tissue samples were dissolved in RIPA as above and, thereafter, all steps were performed as outlined in the kit brochure. Actin, α-actinin, and myosin heavy chain (MHC)-specific antibodies were employed to estimate the total amounts of these proteins. Relative protein carbonylation values were expressed as carbonylation indices (Cls), after normalization for protein amounts.

2.7 In vitro protein carbonylation

Isolated, permeabilized control cardiomyocytes were incubated in a Fenton reaction mixture consisting of FeSO4 (50 μM), hydrogen peroxide (H2O2, 1.5 mM), and ascorbic acid (6 mM) 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 DTT (10 mM, 30 min) to reduce any oxidized SH groups. SH oxidation was investigated by Elmann’s reaction and the oxyblot assay was performed as described previously. Isometric force measurements were performed before and after exposure of the cardiomyocytes to the Fenton reaction and after subsequent DTT treatment (10 mM, 30 min). To investigate the possible interaction between protein...
carbonylation and myofilament phosphorylation, combined treatments were carried out on control cardiomyocytes by application of the Fenton treatment (7 min) before and after PKA exposure (40 min), or after incubation (15 min) with the catalytic subunit of protein phosphatase-1 (PP1c) or -2A (PP2Ac) purified from rabbit skeletal muscle as described.\textsuperscript{27} Ca\textsuperscript{2+}–force relationships were determined before and after the treatments.

2.8 Investigation of SH oxidation

Protein homogenates were prepared as described above for the oxyblot procedure. Each homogenate was divided into two parts. The first part was treated with 10 mM DTT (30 min) in order to reduce the disulfide bridges to SH groups, thereby reversing the SH-dependent modifications. The second part was incubated with relaxing solution only, and was used to compare the SH contents of different myocardial tissue samples. A streptavidin-peroxidase system was used for SH signal detection,\textsuperscript{28} while 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 signals of DTT-treated samples were regarded as 100%.

2.9 Carbonylation of recombinant troponin complexes

Recombinant troponin complexes (Tn) containing troponin T, troponin C, and dephosphorylated or phosphorylated TnI (prepared by Jaquet’s group\textsuperscript{29}) were exposed to Fenton treatment or isolating solution (control) for 7 min to investigate whether TnI phosphorylation affected protein carbonylation. Carbonylation was tested by the oxyblot assay and CIs were compared.

2.10 Statistics

Statistical significance was calculated with the paired Student’s t-test for repeated measurements or one-way analysis of variance followed by a Bonferroni test for multiple comparisons between groups with repeated measurements or one-way analysis of variance followed by a Bonferroni test for multiple comparisons between groups with

3. Results

3.1 Infarct size and global LV function

In Masson’s trichrome-stained LV transversal sections 10 weeks after LAD occlusion, the size of the infarct was 50.3 ± 5.7%. Histological sections in control hearts at identical LV positions exhibited homogeneous staining without signs of infarction (Figure 1). The morphometric data of the control and infarcted mice did not differ significantly (Table 1). However, MI markedly deteriorated the LV systolic function, as evidenced by an almost 50% reduction of EF in the MI group. Moreover, structural differences evidenced by the comparison of the cross-sectional area of isolated cardiomyocytes from the different groups could not be observed (Supplementary material online, Figure S1).

3.2 Mechanical characteristics of isolated cardiomyocytes

Figure 2 demonstrates the results of direct force measurements in permeabilized cardiomyocytes isolated from infarcted and control LVs. The remaining cardiomyocytes in the infarcted area had a lower Ca\textsuperscript{2+} sensitivity of force production (pCa\textsubscript{50}) than that of those in the remote non-infarcted area or that of those in the control left ventricles. These differences in pCa\textsubscript{50} were observed at both shorter and longer SLs (1.9 and 2.3 μm, respectively). However, the length-dependent Ca\textsuperscript{2+} sensitization, i.e., the difference in Ca\textsuperscript{2+} sensitivity in response to sarcomere stretching, was approximately the same in all groups of cardiomyocytes. Moreover, no differences were found in the mean values of F\textsubscript{max}, F\textsubscript{passive}, and k\textsubscript{m, max} or in the visual appearances of the cardiomyocytes from the various myocardial regions (Table 2). The functional characteristics of the cardiomyocytes from the anterior and inferior locations in the non-infarcted control hearts did not differ, and these data were therefore pooled.

3.3 TnI phosphorylation status

To elucidate the molecular background of the decreased Ca\textsuperscript{2+} sensitivity of isometric force production in the cardiomyocytes from the anterior wall, molecular factors formerly implicated as potential modulators of this parameter after MI were systematically analysed. First, the level of TnI phosphorylation (a major determinant of Ca\textsuperscript{2+} sensitivity) was determined at both shorter and longer SLs (1.9 and 2.3 μm, respectively). The functional characteristics of the cardiomyocytes from the anterior and inferior locations in the non-infarcted control hearts did not differ, and these data were therefore pooled.

Figure 1 Histological analysis in Masson’s trichrome-stained LV myocardial sections of a control and an infarcted heart at the level of the papillary muscles. The red staining in the control heart (A) and at the inferior location of the MI heart indicates myocardial tissue without scar formation. Post-MI remodelling at the anterior site (arrows) was reflected by thinning of the myocardial wall and by the accumulation of connective tissue elements (green) in the MI heart (B). Similar observations were made in three hearts.
anterior area than in the controls (Figure 3A–D). Saturation of these PKA-specific phosphorylation sites by in vitro incubation in the presence of the catalytic subunit of PKA resulted in a small, but significant decrease in \( pC_{50} \) in the remaining cardiomyocytes from the MI anterior wall, but not in control cardiomyocytes (Figure 3E–G).

### 3.4 Protein SH oxidation after MI

We next set out to assess the impact of putative oxidative protein modifications on the \( Ca^{2+} \) sensitivity of force production in post-MI cardiomyocytes. In these assays, the SH oxidation of actin was regarded as a marker of myofilament SH oxidation in general. To assess the MI-associated relative SH oxidation in actin, a biotin–streptavidin system was employed. Figure 4A shows the results of a representative western immunoblot assay run on myocardial tissue homogenates from control, MI inferior, and MI anterior LV areas. 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 (Figure 4A and B; Table 3).

In vitro DTT treatment of the protein homogenates increased the reduced SH group content in the homogenates of MI hearts, and hence indicated the reversibility of this oxidative modification. Nevertheless, in vitro DTT
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<thead>
<tr>
<th>Table 1</th>
<th>Morphometric data and EF of control and infarcted mice</th>
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<tbody>
<tr>
<td></td>
<td>Control (n = 5)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>34.6 ± 1.3</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>242 ± 12</td>
</tr>
<tr>
<td>Heart weight/body weight (mg/g)</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>Lung weight/body weight (mg/g)</td>
<td>7.9 ± 0.3</td>
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<tr>
<td>Ejection fraction (%)</td>
<td>62.7 ± 1.3</td>
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</table>

Values are means ± SEM. 
*n*, number of mice. 
*P < 0.05 MI vs. control.

**Figure 2** \( Ca^{2+} \) sensitivity of force production of MI and control cardiomyocytes. \( Ca^{2+} \)–force relationships of MI anterior cardiomyocytes (n = 19) were positioned to the right of those from controls (n = 39) or of those from MI inferior regions (n = 10) at SLs of both 1.9 \( \mu m \) (A) and 2.3 \( \mu m \) (B). The mean \( pC_{50} \) values indicate the decreased \( Ca^{2+} \) sensitivity of force production for the MI anterior cardiomyocytes, but with a preserved length dependence (C). 
*P < 0.05 MI anterior vs. control, *P < 0.05 SL: 1.9 \( \mu m \) vs. SL: 2.3 \( \mu m \).
treatment did not restore the decreased Ca\textsuperscript{2+} sensitivity of force production of MI anterior cardiomyocytes (Figure 4C and D).

3.5 Effect of MI on contractile protein carbonylation

To assess the potential involvement of myofilament protein carbonylation in post-MI remodelling, oxyblot assays were performed in cardiomyocyte protein homogenates from control and infarcted hearts. Figure 5 depicts the results of representative western immunoblots and the means of relative protein carbonylation (expressed as the carbonylation index, where the signal intensity of the control was taken as 1) of MHC, actin, and α-actinin. The degrees of actin and MHC carbonylation were significantly higher at the MI anterior wall than those in the control left ventricle. The differences in the levels of carbonylation of another sarcomeric structural protein, α-actinin, did not reach significance (Table 3). Similarly, no significant differences between control and MI myocytes could be resolved in the carbonylation data at the level of myosin-binding protein C (MyBPC; Table 3).

3.6 Myofilament protein carbonylation decreases Ca\textsuperscript{2+} sensitivity of force production

To verify the hypothetical link between myofilament protein carbonylation and the decreased Ca\textsuperscript{2+} sensitivity of force production, an in vitro Fenton reaction was employed. This involved H\textsubscript{2}O\textsubscript{2}, Fe\textsuperscript{2+}, and ascorbic acid to produce hydroxyl radicals (OH·) for the production of carbonyls in myofilament proteins (Figure 6). Intense protein carbonylation and a limited degree of SH oxidation (relative SH content; control: 100 ± 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 infarcted hearts, enhanced protein carbonylation of actin and MHC was observed in permeabilized control cardiomyocytes following exposure to the Fenton reagents (Figure 6A). Protein carbonylation was not affected by in vitro DTT treatment. Moreover, the levels of relative actin and MHC carbonylation were similar to those observed in the infarcted left ventricle. The application of Fenton solution in isolated control cardiomyocytes at an SL of 2.3 µm resulted in significant decreases in the Ca\textsuperscript{2+} sensitivity of force production and in \( F_p \) (by ~40%) (Figure 6B and C). However, DTT treatment following Fenton treatment failed to reverse the reduced Ca\textsuperscript{2+} sensitivity of control cardiomyocytes. Finally, an apparent linear relationship was seen when the differences between the control \( pC_{50} \) values and those from the MI anterior area, the MI inferior area, and Fenton-treated cardiomyocytes (Δ\( pC_{50} \)) were expressed as a function of the relative protein carbonylation of actin or MHC (Figure 6D and E).

3.7 Myofilament protein carbonylation is independent of myofilament phosphorylation

The effects of Fenton reagents on the Ca\textsuperscript{2+} sensitivity of force production were tested before or after PKA treatments or after protein phosphatase treatments (PP1c or PP2Ac). In accordance with previous results,12,30 PKA exposures had no significant effect on the Ca\textsuperscript{2+} sensitivity in control cardiomyocytes either before or after Fenton treatment, while PP1c decreased, whereas PP2Ac increased the Ca\textsuperscript{2+} sensitivity of force production (Figure 7A–E).31,32 Our results revealed that the functional effect of the Fenton treatment on the Ca\textsuperscript{2+} sensitivity of force production is independent of the phosphorylation status of myofilamentary proteins: the magnitudes of decreases in the mean \( pC_{50} \) values were similar before and after shifting the phosphorylation statuses of myofilament proteins between extreme high and low levels (Figure 7F and F). Further mechanistic insight was obtained at the molecular level of these interactions, when recombinant Tn complexes (with phosphorylated or unphosphorylated TnI subunits) were assayed for protein carbonylations by in vitro Fenton treatment, where TnI phosphorylation did not appear to modulate carbonylations of the protein subunits of the Tn complex (Figure 7G and H).

4. Discussion

This study concentrated on region-specific characteristics of post-MI LV remodelling 10 weeks after 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\textsuperscript{2+} sensitivity of force production was observed, in parallel with pronounced oxidative myofilament protein changes. Likewise, selective experimental protein carbonylation in the control cardiomyocytes provoked a decrease in the Ca\textsuperscript{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 \( pC_{50} \) and the level of protein carbonylation. Hence, we conclude that myofilament protein carbonylation has the potential to reduce the
Figure 3 TnI phosphorylation and PKA-dependent changes in the Ca\(^{2+}\) sensitivity of force production of cardiomyocytes from MI and control hearts. Densitometric analyses (B and D) of western immunoblots (A and C), using phosphorylation-insensitive (TnI) and PKA-specific phosphorylation-sensitive anti-TnI antibodies (TnI-PS\(^{22}\)) (A and B) demonstrated a decreased level of PKA-dependent phosphorylation in the MI anterior area relative to the controls. A PKC-specific (TnI-PT\(^{143}\)) (C and D) antibody did not reveal significant differences in the level of PKC-dependent TnI phosphorylation between MI and control hearts. The TnI amounts determined by phospho-insensitive anti-TnI antibodies (TnI) served normalization purposes. Protein homogenates were from four to five control and six infarcted hearts; assays were repeated three to six times. In vitro PKA administration had no effect on the Ca\(^{2+}\) sensitivity of force production in control cardiomyocytes (n = 4), but further decreased the Ca\(^{2+}\) sensitivity of force production in cardiomyocytes isolated from the infarcted MI anterior region, as illustrated by the significant change in the mean pCa50 value (ΔpCa50) (n = 5) (E–G). *P < 0.05 MI anterior vs control.
Ca\textsuperscript{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. The results of several previous investigations on the cardiomyocyte contractile function, which concentrated on the Ca\textsuperscript{2+} responsiveness of force production in post-MI hearts, suggested that changes in myofilament Ca\textsuperscript{2+} sensitivity (with or without reductions in maximal Ca\textsuperscript{2+}-activated force) play an important role in the LV dysfunction. Nevertheless, no consensus has been reached as concerns the direction and magnitude of the changes in Ca\textsuperscript{2+} sensitivity of force production, as this parameter seems to depend on the combination of species, model, and temporal characteristics of post-infarction remodelling.\textsuperscript{4,5,7,12,33–37} Earlier 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 LAD ligation, we detected no differences in the mechanical functions of cardiomyocytes remote from the infarction and non-infarcted control cardiomyocytes. Moreover, despite the decreased Ca\textsuperscript{2+} sensitivity of force production in the infarcted region, the increase in pCa\textsubscript{50} after sarcomere stretching (from 1.9 to 2.3 \textmu m) was comparable (\textDelta pCa\textsubscript{50} \approx 0.1) in all groups, indicating preservation of the Frank–Starling mechanism in the entire remodelled left ventricle. The possibility that the myocardial remodelling leads to a greater divergence in the mechanical characteristics of the cardiomyocytes at a later time point cannot be excluded.

The Ca\textsuperscript{2+} sensitivity of force production is closely connected with TnI and MyBPC phosphorylation under physiological conditions.\textsuperscript{37,38} A number of previous investigations which sought links between the post-MI myofilament Ca\textsuperscript{2+} sensitivity and TnI phosphorylation revealed that PKA-dependent TnI hypophosphorylation or PKC-dependent TnI hyperphosphorylation may co-segregate with increases or decreases in the Ca\textsuperscript{2+} sensitivity of force production in post-MI hearts.\textsuperscript{4,8} In our model, in vitro PKA exposure resulted in a slight rightward shift in the Ca\textsuperscript{2+}-force relationship of cardiomyocytes from the infarcted region, but not in the control cardiomyocytes. These effects confirm that PKA-

### Table 3 Relative SH oxidation and carbonylation indices of selected myofilament proteins in control and MI hearts

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>MI inferior</th>
<th>MI anterior</th>
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<tr>
<td>Actin SH oxidation (%)</td>
<td>83.8 ± 6.2</td>
<td>62.5 ± 10.3</td>
<td>49.1 ± 10.6*</td>
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<tr>
<td>MHC carbonylation index</td>
<td>1.0 ± 0.07</td>
<td>1.46 ± 0.17</td>
<td>2.06 ± 0.46*</td>
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<tr>
<td>Actin carbonylation index</td>
<td>1.0 ± 0.04</td>
<td>1.40 ± 0.12</td>
<td>1.46 ± 0.18*</td>
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<td>(\alpha)-actinin carbonylation index</td>
<td>1.0 ± 0.07</td>
<td>0.95 ± 0.09</td>
<td>1.25 ± 0.16</td>
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<tr>
<td>MyBPC carbonylation index</td>
<td>1.0 ± 0.14</td>
<td>0.82 ± 0.11</td>
<td>0.70 ± 0.14*</td>
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Protein homogenates derived from four control and seven infarcted hearts; assays were performed in triplicate. Values are means ± SEM.

* \(P < 0.05\) MI anterior vs. control.
mediated TnI phosphorylation is strongly linked with the Ca\(^{2+}\) sensitivity of force production.\(^{15,39}\) 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.

Oxidative post-translational protein modifications can potentially contribute to the development of post-MI remodelling, since inflammation is a main pathogenic factor in this process.\(^{40,41}\) Myofilament proteins may suffer from numerous types of oxidative modifications, including SH oxidation,\(^{42}\) OH--mediated irreversible carboxylation of lysine, arginine, and proline residues,\(^{43}\) or peroxynitrite-evoked tyrosine nitration\(^{44}\) during MI. These protein modifications contribute to the LV dysfunction.\(^{45}\) For example, SH oxidation of tropomyosin,\(^{9}\) and/or of actin in combination with myosin light chain 1,\(^{28}\) and the nitration of \(\alpha\)-actinin\(^{46}\) have been suggested as potential mediators of oxidative myocardial contractile depression. Importantly, only myofilament SH oxidation, but not protein nitration, was found to be associated with decreased Ca\(^{2+}\) sensitivity of force production, and oxidative changes affecting myofilamental SH groups (and their mechanical consequences) could be effectively reversed by \textit{in vitro} antioxidant DTT treatment.\(^{28}\) In our present study, we observed increased levels of SH oxidation in cardiomyocytes from the infarcted region. Interestingly, reduction of the oxidized SH groups by \textit{in vitro} DTT treatment was not paralleled by a restoration of the myofilament Ca\(^{2+}\) sensitivity to the control level. We, therefore, postulate that the relatively small degree of SH oxidation in the cardiomyocytes from the infarcted region was not responsible for the decreased Ca\(^{2+}\) sensitivity of force production in the cardiomyocytes from the infarcted anterior region.

Protein carbonylation is widely accepted as a marker of severe oxidative stress, as it has been detected in a broad range of human diseases, including diabetes, arteriosclerosis, respiratory, and renal failure.\(^{47}\) An enhanced level of carbonylation of skeletal MHC was reported to be associated with a reduced myosin sliding velocity in \textit{in vitro} motility studies.\(^{48}\) Enhanced myofibrillar protein carbonylation has also been documented for post-ischaemic reperfusion\(^{22}\) and in studies involving a model of coronary microembolization.\(^{9}\) Canton \textit{et al.}\(^{9}\) reported that actin and tropomyosin are specifically sensitive to this type of irreversible protein modification in canine and porcine models involving coronary microembolization, where the extents of actin and tropomyosin carbonylation correlated inversely with the systolic wall thickening. Moreover, the same authors extended their investigations to myofibrillar carbonylation in human heart failure, and found that increased levels of actin and tropomyosin carbonylation were accompanied by a reduction in LV EF.\(^{49}\) Besides the contractile machinery, oxidative insults on the cellular Ca\(^{2+}\) homeostasis might also contribute to the LV contractile dysfunction, through

**Figure 5** Relative levels of contractile protein carbonylation after MI. The results of the oxyblot assay suggested higher levels of MHC and actin carbonylation in cardiomyocytes from the anterior LV region of the MI hearts than in the controls. The carbonylation level of \(\alpha\)-actinin appeared to be similar in all three groups of cardiomyocytes. Protein amounts determined by MHC, actin, or \(\alpha\)-actinin-specific primary antibodies (protein) in western blots served normalization purposes for calculation of carbonylation indices. Protein homogenates were from four control and seven infarcted hearts; assays were performed in triplicate. *\(P < 0.05\) MI anterior vs. control.
Figure 6 In vitro carbonylation of myofilament proteins decreased the Ca\textsuperscript{2+} sensitivity of force production in control cardiomyocytes. Incubation of cardiomyocytes in the presence of Fenton reagent increased the carbonylation levels of several myocardial proteins, including MHC and actin (A). The carbonylation levels were not influenced following exposure to 10 mM DTT. Cardiomyocytes were from four control murine hearts; assays were performed in quadruplicate. Actin and MHC protein amounts determined with the use of anti-actin and anti-MHC antibodies (MHC, Actin) in western blots served normalization purposes. The enhanced protein carbonylation of the control cardiomyocytes shifted the Ca\textsuperscript{2+}–force relationship to the right (B) and significantly decreased its mean pCa\textsubscript{50} value (pCa\textsubscript{50} changed from 5.76 ± 0.03 to 5.62 ± 0.04) (C). Subsequent DTT treatment (Fenton + DTT) did not restore the Ca\textsuperscript{2+} sensitivity of force production (n = 7 cardiomyocytes from three mouse hearts) (B and C). *p < 0.05 vs. control. Positive correlations were found between the changes in Ca\textsuperscript{2+} sensitivity of force production and the degree of actin (D) (p = 0.0264, r\textsuperscript{2} = 0.8661) or MHC carbonylation (p = 0.0114, r\textsuperscript{2} = 0.9117) both in MI hearts and following in vitro carbonyl group induction (Fenton) (E).
Figure 7  The effect of protein carbonylation on Ca\textsuperscript{2+} sensitivity of force production is independent of the phosphorylation status of myofilament proteins. Incubation of control cardiomyocytes in the presence of the catalytic subunit of PKA did not affect the Ca\textsuperscript{2+} sensitivity significantly either before (A and E) or after Fenton treatment (B and E), which decreased the Ca\textsuperscript{2+} sensitivity (n = 6–5 cardiomyocytes from three mouse hearts). PP1c decreased (C and E), while PP2Ac increased the Ca\textsuperscript{2+} sensitivity (D and E), and the subsequent Fenton reaction resulted in lower Ca\textsuperscript{2+} sensitivities of force production in both cases (n = 6–6 cardiomyocytes from three mouse hearts). The carbonylation-dependent decreases in Ca\textsuperscript{2+} sensitivities of force production were reflected by lower pCa\textsubscript{50} values relative to the levels preceding the Fenton treatment (E). The decreases in pCa\textsubscript{50} values were similar before and after the different treatments (F). The three subunits of recombinant Tn complexes (Tn with phosphorylated or dephosphorylated TnI—confirmed with phospho-specific antibodies—TnI-PS22) showed similar increases in their levels of carbonylation independently of the TnI phosphorylation status (G and H). *P < 0.05 before vs. after Fenton; **P < 0.05 before vs. after PP1c or PP2Ac.
modulation of the intracellular Ca\textsuperscript{2+}-handling proteins.\textsuperscript{50} In the present study, increased levels of actin and MHC carbonylation were observed in cardiomyocytes from the infarcted region of murine hearts 10 weeks after LAD ligation, but not in cardiomyocytes of remote regions. These findings implicated a potential pathophysiologic role for protein carbyl- nation in the surviving cardiomyocytes of the infarcted region. Moreover, actin and MHC carbonylation could also be provoked through in vitro test incubations in the presence of OH- radicals. The in vitro tests with Fenton reagents suggested a carbonylation-dependent reduction in \( F_0 \), but this change was not reflected in the surviving cardiomyocytes of the anterior region of our in vivo infarction model. Although the literature data on post-ischaemic changes in \( F_0 \) do not appear to be consistent,\textsuperscript{4,7} we hypothesize that part of the above discrepancy might be related to differ-
tential carbonylation of myofilament proteins determining \( F_0 \) under in vitro and in vivo conditions.

The magnitude of change in Ca\textsuperscript{2+} sensitivity of force production was similar irrespective of the phosphorylation status of the myofilament proteins. The results of previous studies\textsuperscript{31,32} indicated that \( \Delta F_0 \) decreases, and \( \Delta P_{Ca_{50}} \) increases the Ca\textsuperscript{2+} sensitivity of force production through the predominant dephosphorylation of myosin light chain 2 or TnI, respectively. However, subsequent carbonylation resulted in similar degrees of decreases in the Ca\textsuperscript{2+} sensitivity of force production in both of the above cases. Hence, the mechanical effect of protein carbonyla-
tion was independent of myofilament phosphorylation. Experiments on recombinant Tn complexes further supported the former hypoth-
thesis, where similar degrees of carbonylation could be observed in all three Tn subunits irrespective of the PKA-dependent phosphorylation status of TnI. Interestingly, the carbonylation of myofilament proteins was associated with a decreased Ca\textsuperscript{2+} sensitivity of force production under both in vitro and in vivo conditions, and a positive correlation was found between \( \Delta p_{Ca_{50}} \) and the degree of protein carbonylation. We, therefore, propose that myofilbrar protein carbonylation can be a major determinant of post-MI contractile dysfunction in the remaining myocardium of the infarcted zone. Nevertheless, the contribution of other mechanisms cannot be excluded.

Overall, our data revealed that the protein carbonylation induced by experimental MI is associated with decreased myofilament Ca\textsuperscript{2+} sensi-
tivity in the surviving cardiomyocytes of the infarcted zone. It additionally emerged that the effects of other mechanisms that affect the Ca\textsuperscript{2+} sensi-
tivity (such as PKA-dependent TnI hypophosphorylation) may be over-
ridden by this irreversible of protein modification. The reduction in Ca\textsuperscript{2+} sensitivity of force production renders the surviving myocardium in the infarcted zone hypocontractile, even if the cardiomyocyte Ca\textsuperscript{2+} homeostasis is preserved.

## Supplementary material

Supplementary material is available at Cardiovascular Research online.

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## Conflict of interest

None declared.

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