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

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Abstract

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) remodeling 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 noninfarcted inferior LV area were compared with those of noninfarcted age-matched controls. Myofilament phosphorylation, sulfhydryl (SH) oxidation and carbonylation were also assayed. The Ca^{2+} sensitivity of force production was significantly lower in the anterior wall (pCa_{50}:5.81±0.03, mean±SEM, at 2.3 μm sarcomere length) than that in the controls (pCa_{50}:5.91±0.02) or in the MI inferior area (pCa_{50}: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 Ca^{2+} 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_{MHC}:2.06±0.46, CI_{actin}:1.46±0.18] than in the controls (*CI*:1). *In vitro* Fenton-based myofilament carbonylation in the control cardiomyocytes also decreased the Ca^{2+} sensitivity of force production irrespective of the phosphorylation status of the myofilaments. Furthermore, the Ca^{2+} sensitivity correlated strongly with myofilament carbonylation levels in all investigated samples. Conclusions: Post-MI myocardial remodeling involves increased myofibrillar protein carbonylation and decreased Ca^{2+} sensitivity of force production, leading potentially to contractile dysfunction in the remaining cardiomyocytes of the infarcted area.

Keywords: contractile function, infarction, myocytes, remodeling, sarcomere

Introduction

A large number of cardiomyocytes die via apoptosis[1] and necrosis during myocardial infarction (MI), and the remaining cardiomyocytes often enter a deleterious remodeling process leading to cardiac dysfunction. The functional cardiomyocyte changes involve the intracellular Ca^{2+} handling,[2] and Ca^{2+} sensitivity of force production of the contractile machinery in the remodeled heart.[3] Previous publications have reported various, often conflicting post-MI-linked myofilamentary alterations. For example, the Ca^{2+} responsiveness of the contractile protein machinery was found to be either decreased,[4, 5] unaltered[6, 7] or increased.[3, 8] Different molecular alterations, mostly posttranslational myofibrillar protein modifications have been suggested for the explanation of the above mechanical abnormalities.[3, 9, 10] In this context, prolonged neurohumoral activation with consequent maladaptive compensatory changes at the level of the cardiomyocytes (e.g. down-graded β-adrenergic signaling) may potentially lead to altered protein phosphorylation.[11] A pathogenic role for protein kinase A (PKA)-dependent cardiac troponin I (TnI) hypophosphorylation[8, 12-15] or increased protein kinase C (PKC)-mediated myofilament protein phosphorylation[16] have been implicated during this process. Moreover, inflammatory processes, e.g. the participation of reactive oxygen and nitrogen species (ROS/RNS) leading to oxidative myofilament protein alterations, may complement the above signaling pathways.[17-21] Indeed, Canton et al. reported on the oxidation of actin and tropomyosin in postischemic rat hearts[22], and tropomyosin oxidation was demonstrated in porcine and canine myocardial preparations after coronary microembolizations.[9] Interestingly, the level of tropomyosin oxidation correlated inversely with the contractile function in postischemic porcine hearts,[9] suggesting that oxidative myofilament alterations might be responsible, at least in part, for the postischemic myocardial dysfunction.

Myocardial ischemia develops predominantly in myocardial regions directly supplied by the occluded coronary arteries, yet MI-related left ventricular (LV) remodeling may also involve remote regions.[23] 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.

We have now investigated the region-specific characteristics of postischemic myocardial remodeling in a mouse model of MI. Direct force measurements in permeabilized cardiomyocytes allowed a comparison of the post-MI remodeling in the infarcted anterior and noninfarcted inferior LV areas in murine hearts 10 weeks after ligation of the left anterior descending coronary artery (LAD), 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 postischemic mechanical dysfunction.

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

Methods

Detailed materials and methods are provided in the Supplementary material online.

Animals

Female OF-1 mice (32.9±0.5 g, 16 weeks old, *n*=30) were anesthetized with intraperitoneal (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). Postoperatively, the mice received

analgesic treatment (buprenorphin, 0.1 mg/kg, in the drinking water). Noninfarcted hearts from sham-operated animals served as controls (*n*=5). All procedures conform to the Directive 2010/63/EU of the European Parliament and were approved by the animal ethics committee of the Medical University of Vienna (GZ: 66.009/0173-II/10b/2009).

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 2-dimensional parasternal short-axis view of the LV at the mid-papillary muscle level.

Infarct size determination

Under anesthesia (see above), beating hearts were excised and were either fixed in 4% formaldehyde for histologic analyses, or dissected for later mechanical cardiomyocyte measurements or for biochemical assays: the infarcted area was separated from the noninfarcted, remote area of the LV. Samples were weighed and stored at -80 °C. Infarct size was determined in Masson's trichrome-stained LV sections as described previously.[24]

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.[25] 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 a SL of 2.3 μm. Isometric force values were normalized for the maximal Ca^{2+}-activated active force, and Ca^{2+}–force relations were fitted to a modified Hill equation to determine the Ca^{2+} sensitivity of isometric

force production, i.e. pCa_{50}. Active isometric force (F_o), Ca^{2+}-independent passive force ($F_{passive}$) and the rate tension of force redevelopment ($k_{tr,max}$) were determined.

Additionally, the effect of PKA (catalytic subunit of bovine heart PKA; Sigma-Aldrich, St. Louis, MO, USA) on pCa_{50} was also measured. To assess the mechanical effects of sulfhydryl (SH) oxidation, antioxidant treatment (10 mM DTT) was applied. Ca^{2+}–force relationships were determined at a SL of 2.3 μm before and after *in vitro* PKA (40 min) or DTT treatment (30 min).

Investigation of the phosphorylation status of cardiac TnI

A modified RIPA (radio-immunoprecipitation assay) 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%.

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 (*CI*), after normalization for protein amounts.

***In vitro* protein carbonylation**

Isolated, permeabilized control cardiomyocytes were incubated in a Fenton reaction mixture consisting of $FeSO_4$ (50 μM), hydrogen peroxide (H_2O_2, 1.5 mM) and ascorbic acid (6 mM) for 7 min at room temperature,[26] either in the mechanical set-up or before oxyblot assays in

test-tubes. A subset of the samples was treated with DTT (10 mM) to reduce any oxidized SH groups. SH oxidation was investigated by Ellmann's reaction and 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[27]. Ca^{2+}–force relationships were determined before and after the treatments.

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,[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%.

Carbonylation of recombinant troponin complexes

Recombinant troponin complexes (Tn) containing troponin T (TnT), troponin C (TnC) and dephosphorylated or phosphorylated TnI (prepared by Jaquet's group[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 oxyblot assay and carbonylation indices were compared.

Statistics

Statistical significance was calculated with the paired Student t test for repeated measurements or one-way analysis of variance followed by a Bonferroni test for multiple comparisons between groups with $P<0.05$. 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. Values are given as means±SEM.

Results

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 (Fig. 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 (Suppl. Fig. 1).

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^{2+} sensitivity of force production (pCa_{50}) than that of those in the remote noninfarcted area or that of those in the control LVs. These differences in pCa_{50} were observed at both shorter and longer SLs (1.9 μm and 2.3 μm, respectively). However, the length-dependent Ca^{2+} sensitization, i.e. the difference in Ca^{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_o, $F_{passive}$ and $k_{tr,max}$ 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 noninfarcted control hearts did not differ, and these data were therefore pooled.

Troponin I phosphorylation status

To elucidate the molecular background of the decreased Ca^{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 analyzed. First, the level of TnI phosphorylation (a major determinant of Ca^{2+} sensitivity) was investigated (Fig. 3). The levels of TnI phosphorylation at the PKA- and PKC-specific sites were assessed in Western immunoblot assays by using phospho-specific TnI antibodies. The extent of phosphorylation at a PKC-specific (threonine 143) site did not differ significantly among the three groups. However, a PKA-specific (serine 22) site of TnI was less extensively phosphorylated in the cardiomyocytes in the infarcted anterior area than in the controls (Fig. 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 pCa_{50} in the remaining cardiomyocytes from the MI anterior wall, but not in control cardiomyocytes (Fig. 3e-g).

Protein SH oxidation after myocardial infarction

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.[28] 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 (Fig. 4a,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 treatment did not restore the decreased Ca^{2+} sensitivity of force production of MI anterior cardiomyocytes (Fig. 4c,d).

Effect of myocardial infarction on contractile protein carbonylation

To assess the potential involvement of myofilament carbonylation in post-MI remodeling, 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 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 LV. 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).

Myofilament protein carbonylation decreases Ca^{2+} sensitivity of force production

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. This involved H_2O_2, Fe^{2+} and ascorbic acid to produce hydroxyl radicals (OH·) for the production of carbonyls in myofilament proteins (Fig. 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 (Fig. 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 LVs. The application of Fenton solution in isolated control cardiomyocytes at a SL of 2.3 μm resulted in significant decreases in the Ca^{2+} sensitivity of force production and in F_o (by about 40%) (Fig. 6b,c). However, DTT treatment following Fenton treatment failed to reverse the reduced Ca^{2+} sensitivity of control cardiomyocytes. 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 or MHC (Fig. 6d,e).

Myofilament protein carbonylation is independent of myofilament phosphorylation

The effects of Fenton reagents on the Ca^{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, PKA exposures had no significant effect on the Ca^{2+} sensitivity in control cardiomyocytes[12, 30] either before or after Fenton treatment, while PP1c decreased whereas PP2Ac increased the Ca^{2+} sensitivity of force production (Fig. 7a-e).[31, 32] 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 myofilamentary proteins: the magnitude of decreases in the mean pCa_{50} values were similar before and after shifting the phosphorylation statuses of myofilament proteins between extreme high and low levels (Fig. 7e,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 (Fig. 7g,h).

Discussion

This study concentrated on region-specific characteristics of post-MI LV remodeling 10 weeks after LAD ligation in mice. Cardiomyocytes from the infarcted anterior and from the noninfarcted 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. 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.

The results of several previous investigations on the cardiomyocyte contractile function, which concentrated on the Ca^{2+} responsiveness of force production in post-MI hearts, suggested that changes in myofilament Ca^{2+} sensitivity (with or without reductions in maximal Ca^{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^{2+} sensitivity of force production, as this parameter seems to depend on the combination of species, model and temporal characteristics of postinfarction remodeling.[4, 5, 7, 13, 33-37] Earlier studies focused primarily on noninfarcted areas of the left and/or right ventricles, whereas we investigated both the MI-affected and noninfarcted LV regions. At 10 weeks after LAD

ligation, we detected no differences in the mechanical functions of cardiomyocytes remote from the infarction and noninfarcted control cardiomyocytes. Moreover, despite the decreased Ca^{2+} sensitivity of force production in the infarcted region, the increase in pCa_{50} after sarcomere stretching (from 1.9 μm to 2.3 μm) was comparable (ΔpCa_{50}~0.1) in all groups, indicating preservation of the Frank-Starling mechanism in the entire remodeled LV. The possibility that the myocardial remodeling leads to a greater divergence in the mechanical characteristics of the cardiomyocytes at a later time point cannot be excluded.

The Ca^{2+} sensitivity of force production is closely connected with TnI and MyBPC phosphorylation under physiological conditions.[37, 38] A number of previous investigations which sought links between the post-MI myofilament Ca^{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^{2+} sensitivity of force production in post-MI hearts.[4, 8] In our model, *in vitro* PKA exposure resulted in a slight rightward shift in the Ca^{2+}–force relations of cardiomyocytes from the infarcted region, but not in the control cardiomyocytes. These effects confirm that PKA-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 posttranslational protein modifications can potentially contribute to the development of post-MI remodeling 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 carbonylation 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 α-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 *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 *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 *in vitro* motility studies.[48] Enhanced myofibrillar protein carbonylation has also been documented for postischemic reperfusion[22] and in studies involving a model of coronary microembolization.[9] Canton et al. 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.[9] 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 ejection fraction.[49]

Besides the contractile machinery, oxidative insults on the cellular Ca^{2+} homeostasis might also contribute to the LV contractile dysfunction, through modulation of the intracellular Ca^{2+} handling proteins.[50] In the present study, increased levels of actin and MHC carbonylation were observed in cardiomyoctes from the infarcted region of murine hearts 10 weeks after LAD ligation, but not in cardiomyocytes of remote regions. These findings implicated a potential pathophysiological role for protein carbonylation 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_o, but this change was not reflected in the surviving cardiomoycytes of the anterior region of our *in vivo* infarction model. While the literature data on postischemic changes in F_o do not appear to be consistent,[3, 4, 7] we hypothesize that part of the above discrepancy might be related to differential carbonylation of myofibrillar proteins determining F_o under *in vitro* and *in vivo* conditions.

The magnitude of change in Ca^{2+} sensitivity of force production was similar irrespective of the phosphorylation status of the myofilament proteins. The results of previous studies[31, 32] indicated that PP1c decreases, and PP2Ac increases the Ca^{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^{2+} sensitivity of force production in both of the above cases. Hence, the mechanical effect of protein carbonylation was independent of myofilament phosphorylation. Experiments on recombinant Tn complexes further supported the former hypothesis, 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^{2+} sensitivity of force production under both *in vitro* and *in vivo*

conditions, and a positive correlation was found between ΔpCa_{50} and the degree of protein carbonylation. We therefore propose that myofibrillar 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^{2+} sensitivity in the surviving cardiomyocytes of the infarcted zone. It additionally emerged that the effects of other mechanisms that affect the Ca^{2+} sensitivity (such as PKA-dependent TnI hypophosphorylation) may be overridden by this irreversible type of protein modification. The reduction in Ca^{2+} sensitivity of force production renders the surviving myocardium in the infarcted zone hypocontractile, even if the cardiomyocyte Ca^{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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Figure legends

Fig. 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 remodeling 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 3 hearts

Fig. 2 Ca^{2+} sensitivity of force production of MI and control cardiomyocytes. Ca^{2+}–force relations 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 sarcomere lengths (SLs) of both 1.9 μm (**a**) and 2.3 μm (**b**). The mean pCa_{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 μm *vs* SL: 2.3 μm

Fig. 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 ($TnI\text{-}P^{S22}$) (**a** and **b**) demonstrated a decreased level of PKA-dependent phosphorylation in the MI anterior area relative to the controls. A PKC-specific ($TnI\text{-}P^{T143}$) (**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 4-5 control and 6 infarcted hearts; assays were repeated 3-6 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; $^{\#}P$<0.05 before *vs.* after PKA

Fig. 4 Relative levels of myofilament protein SH oxidation after MI. Western immunoblot assays (**a**) combined with densitometric analyses (**b**) revealed a higher degree of SH oxidation in sarcomeric actin from the MI anterior cardiomyocytes than that in the control cardiomyocytes. Actin amounts determined with the use of anti-actin primary antibodies (*Actin*) served normalization purposes. 10 mM DTT reduced all oxidized SH groups of cardiomyocytes (signal intensity: 100%), but did not affect the Ca^{2+} sensitivity of force production in isolated MI anterior cardiomyocytes (n=6) (**c**), as illustrated by the similar mean pCa_{50} values (pCa_{50}*(before DTT)*: 5.79±0.02, pCa_{50}*(after DTT)*: 5.76±0.02; P>0.05) (**d**). Protein homogenates were from 4 control and 7 infarcted hearts; assays were performed in triplicate. **P*<0.05 MI anterior *vs.* control, $^{\#}P$<0.05 before *vs.* after DTT within the same group

Fig. 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 α-actinin appeared to be similar in all three groups of cardiomyocytes. Protein amounts determined by MHC, actin or α-actinin-specific primary antibodies (*protein*) in Western blots served normalization purposes for calculation of carbonylation indices. Protein homogenates were from 4 control and 7 infarcted hearts; assays were performed in triplicate. **P*<0.05 MI anterior *vs.* control

Fig. 6 *In vitro* carbonylation of myofilament proteins decreased the Ca^{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 4 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^{2+}–force relation to the right (**b**) and significantly decreased its mean pCa_{50} value (pCa_{50} changed from 5.76±0.03 to 5.62±0.04) (**c**). Subsequent DTT treatment (*Fenton+DTT*) did not restore the Ca^{2+} sensitivity of force production (n=7 cardiomyocytes from 3 mouse hearts) (**b** and **c**). *P<0.05 *vs.* control. Positive correlations were found between the changes in Ca^{2+} sensitivity of force production and the degree of actin (**d**) (P=0.0264, r^2=0.8661) or MHC carbonylation (P=0.0114, r^2=0.9117) both in MI hearts and following *in vitro* carbonyl group induction (*Fenton*) (**e**)

Fig. 7 The effect of protein carbonylation on Ca^{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^{2+} sensitivity significantly either before (**a** and **e**) or after Fenton treatment (**b** and **e**), which decreased the Ca^{2+} sensitivity (n=6-5 cardiomyocytes from 3 mouse hearts). PP1c decreased (**c** and **e**), while PP2Ac increased the Ca^{2+} sensitivity (**d** and **e**), and the subsequent Fenton reaction resulted in lower Ca^{2+} sensitivities of force production in both cases (n=6-6 cardiomyocytes from 3 mouse hearts). The carbonylation-dependent decreases in Ca^{2+} sensitivities of force production were reflected by lower pCa_{50} values relative to the levels

preceding the Fenton treatment (**e**). The decreases in pCa_{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-P^{S22}) 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

Table 1 Morphometric data and ejection fraction of control and infarcted mice

Values are means±SEM. (*n*=number of mice; **P*<0.05 MI *vs.* control)

Table 2 Mechanical characteristics of cardiomyocytes from control and infarcted hearts

Values are means±SEM. (*n*=number of cardiomyocytes from 5 control and 8 infarcted hearts; **P*<0.05 SL: 1.9 µm *vs.* SL: 2.3 µm; $^{\#}P<0.05$ MI anterior *vs.* control)

Table 3 Relative SH oxidation and carbonylation indices of selected myofilament proteins in control and MI hearts. Protein homogenates derived from 4 control and 7 infarcted hearts; assays were performed in triplicate. Values are means±SEM. **P*<0.05 MI anterior *vs.* control

Table 1

	control (*n*=5)	**MI** (*n*=8)
Body weight (g)	34.6 ± 1.3	37 ± 0.8
Heart weight (mg)	242 ± 12	295 ± 25
Heart weight/body weight (mg/g)	7.0 ± 0.4	7.9 ± 0.5
Lung weight/body weight (mg/g)	7.9 ± 0.3	8.2 ± 0.3
Ejection fraction (%)	62.7 ± 1.3	34.9 ± 3.6*

Table 2

	control (*n*=39)		**MI inferior** (*n*=10)	
SL	1.9 µm	2.3 µm	1.9 µm	2.3 µm
F_o **(kN/m^2)**	9.53 ± 0.55	15.66 ± 1.27*	11.52 ± 1.42	15.91 ± 2.26*
$F_{passive}$ **(kN/m^2)**	0.36 ± 0.04	2.23 ± 0.25*	0.60 ± 0.11	2.41 ± 0.52*
pCa_{50}	5.81 ± 0.02	5.91 ± 0.02*	5.80 ± 0.02	5.88 ± 0.02*
n_{Hill}	2.08 ± 0.06	1.63 ± 0.05	2.20 ± 0.17	1.71 ± 0.08
$k_{tr,max}$ **(1/sec)**	3.69 ± 0.15	3.80 ± 0.12	4.08 ± 0.40	4.31 ± 0.21

Table 3

	control	MI inferior
Actin SH oxidation (%)	83.8 ± 6.2	62.5 ± 10.3
MHC carbonylation index	1.0 ± 0.07	1.46 ± 0.17
Actin carbonylation index	1.0 ± 0.04	1.40 ± 0.12
α-actinin carbonylation index	1.0 ± 0.07	0.95 ± 0.09
MyBPC carbonylation index	1.0 ± 0.14	0.82 ± 0.11

Figure(s)

Fig. 1

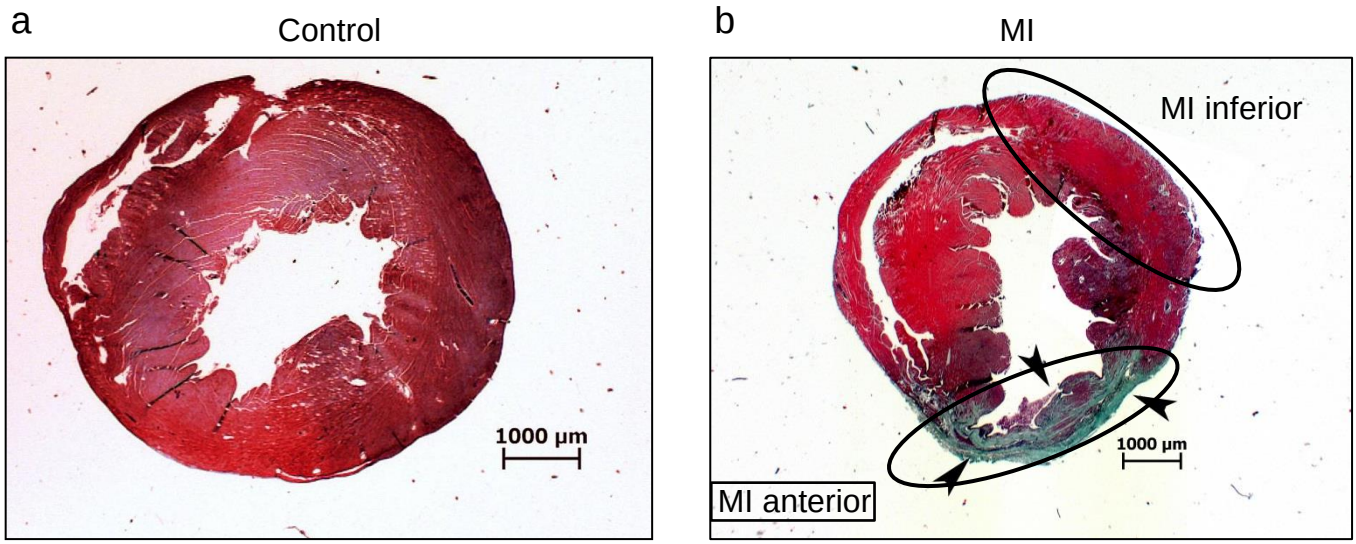


Fig. 2

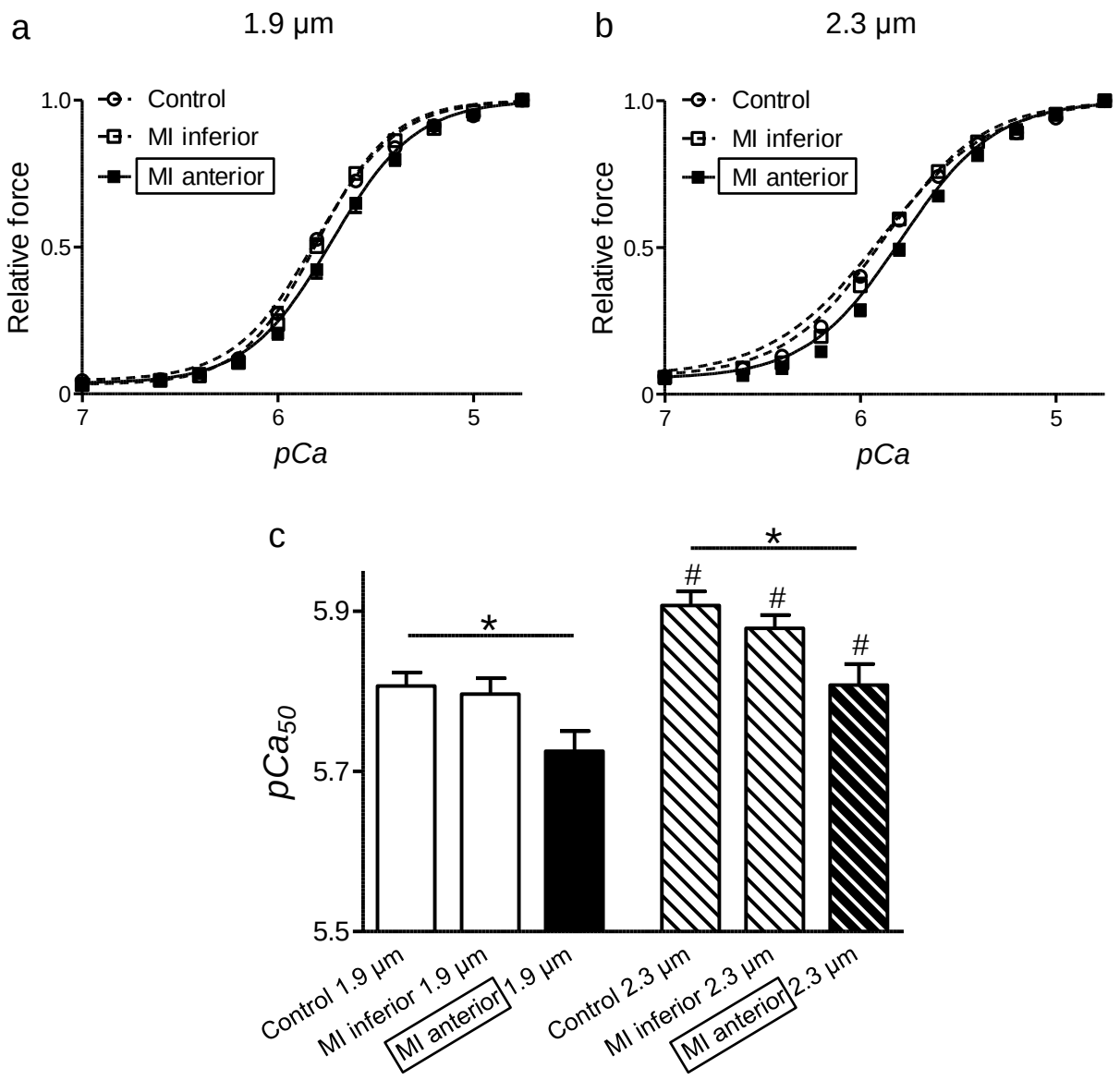

a
1.9 µm
Control
MI inferior
MI anterior
Relative force
1.0
0.5
0
7
6
5
pCa
b
2.3 µm
Control
MI inferior
MI anterior
Relative force
1.0
0.5
0
7
6
5
pCa
c
pCa50
5.9
5.7
5.5
*
*
#
#
#
Control 1.9 µm
MI inferior 1.9 µm
MI anterior 1.9 µm
Control 2.3 µm
MI inferior 2.3 µm
MI anterior 2.3 µm

Fig. 3

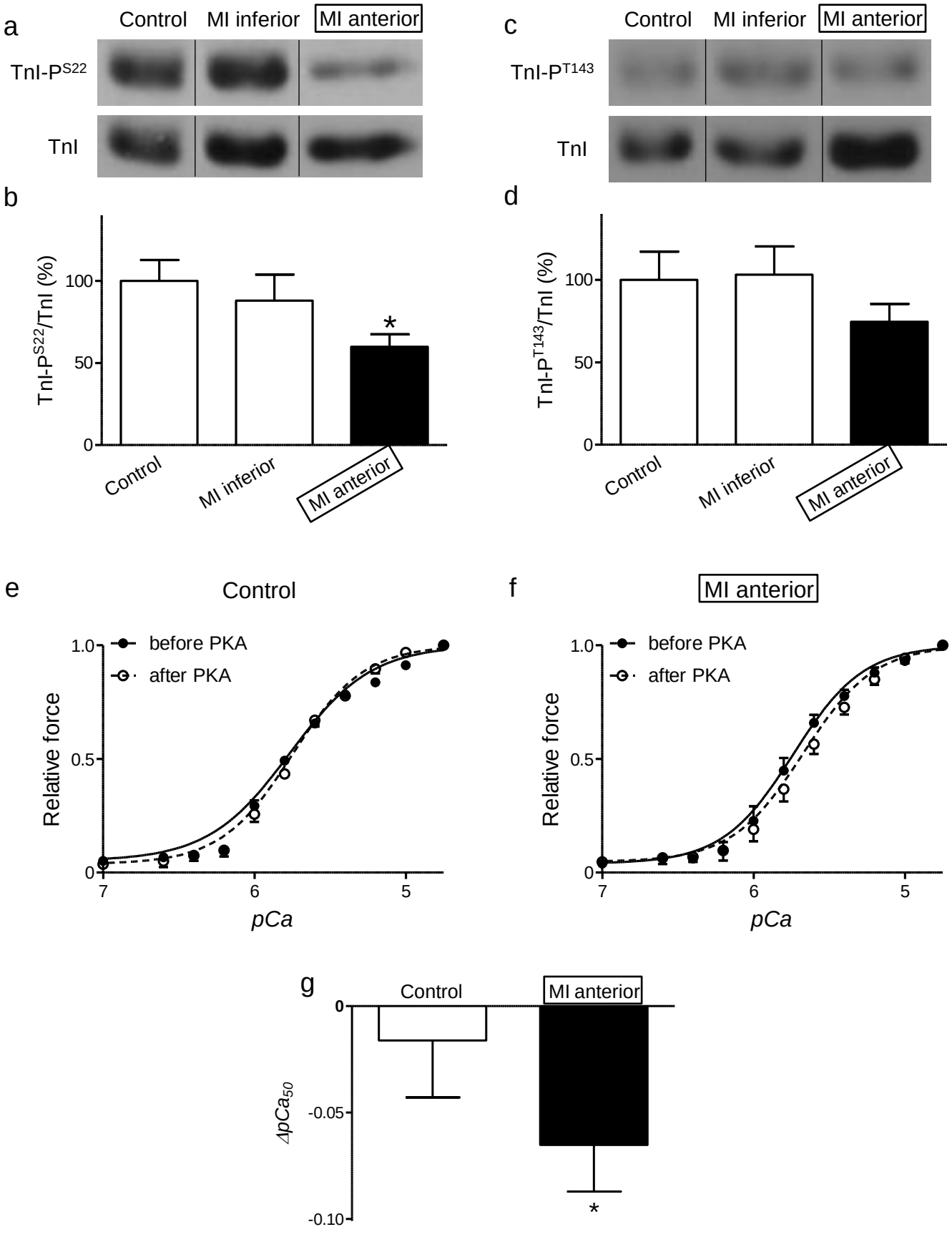

a
Control
MI inferior
MI anterior
TnI-P^S22
TnI
c
Control
MI inferior
MI anterior
TnI-P^T143
TnI
b
TnI-P^S22/TnI (%)
100
50
0
*
Control
MI inferior
MI anterior
d
TnI-P^T143/TnI (%)
100
50
0
Control
MI inferior
MI anterior
e
Control
before PKA
after PKA
Relative force
1.0
0.5
0
7
6
5
pCa
f
MI anterior
before PKA
after PKA
Relative force
1.0
0.5
0
7
6
5
pCa
g
Control
MI anterior
0
-0.05
-0.10
ΔpCa50
*

Fig. 4

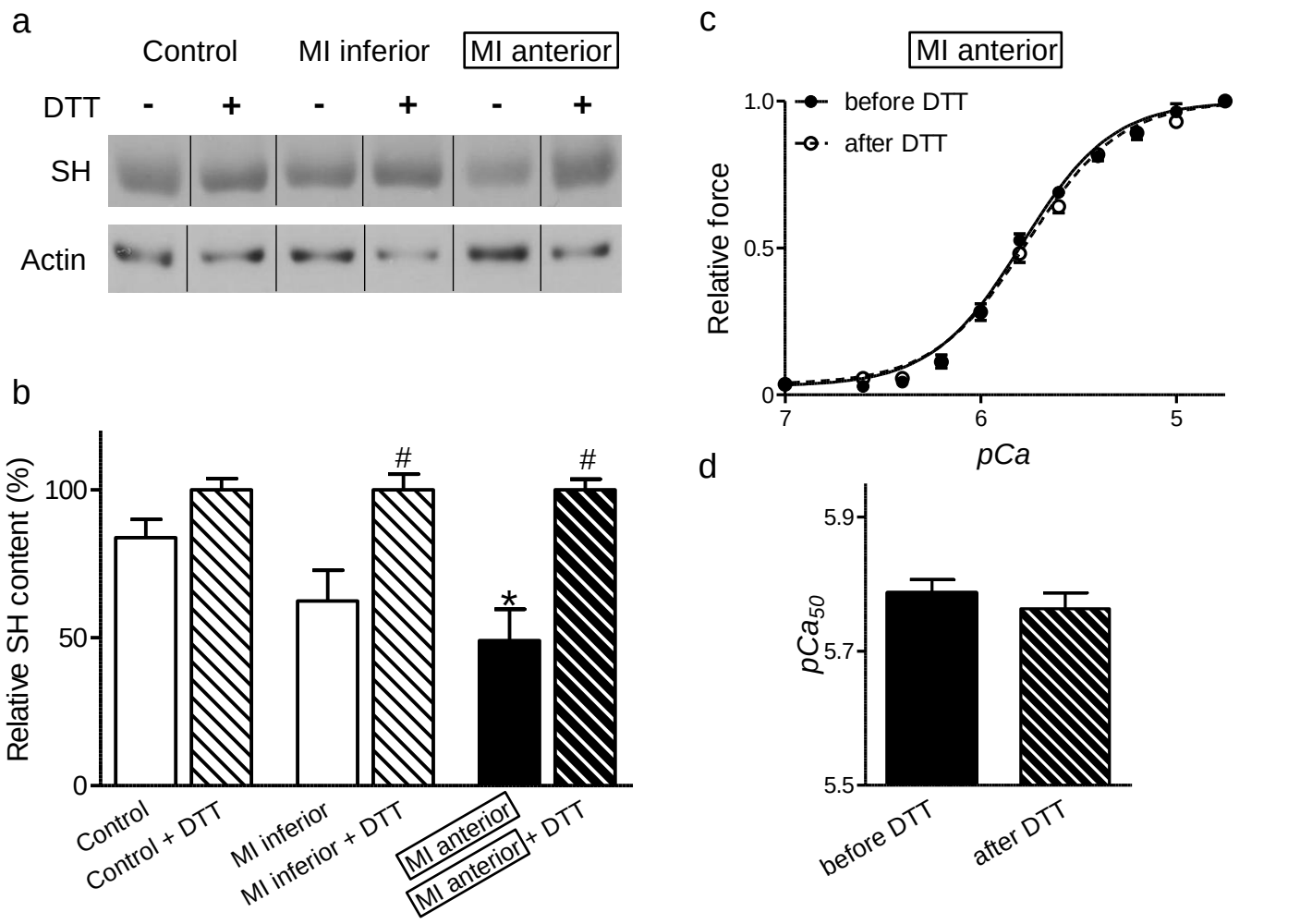

a
Control
MI inferior
MI anterior
DTT
- + - + - +
SH
Actin
b
Relative SH content (%)
100
50
0
#
#
*
Control
Control + DTT
MI inferior
MI inferior + DTT
MI anterior
MI anterior + DTT
c
MI anterior
before DTT
after DTT
Relative force
1.0
0.5
0
7
6
5
pCa
d
pCa50
5.9
5.7
5.5
before DTT
after DTT

Fig. 5

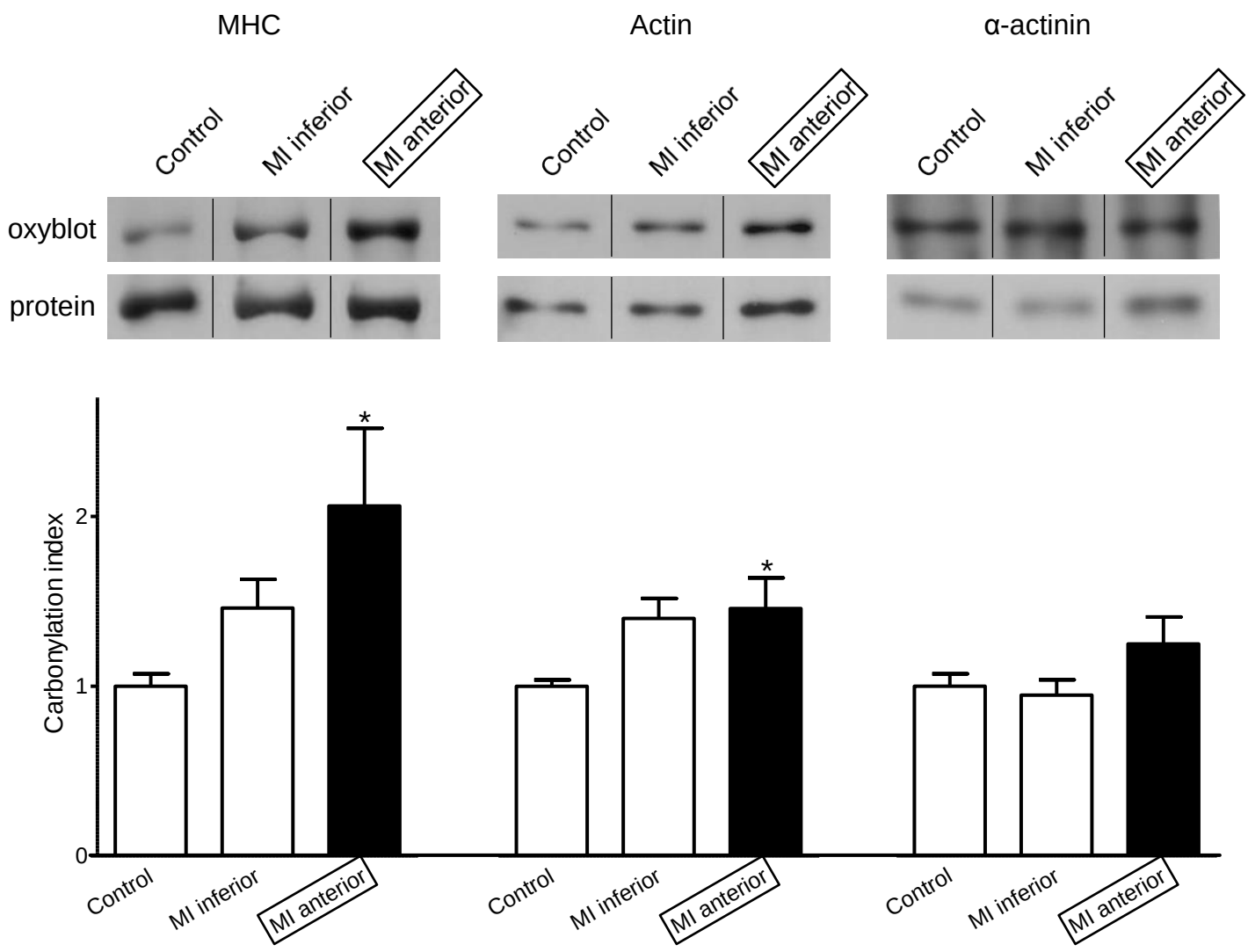

MHC
Actin
α-actinin
Control
MI inferior
MI anterior
oxyblot
protein
Carbonylation index
0
1
2
*
*

Fig. 6

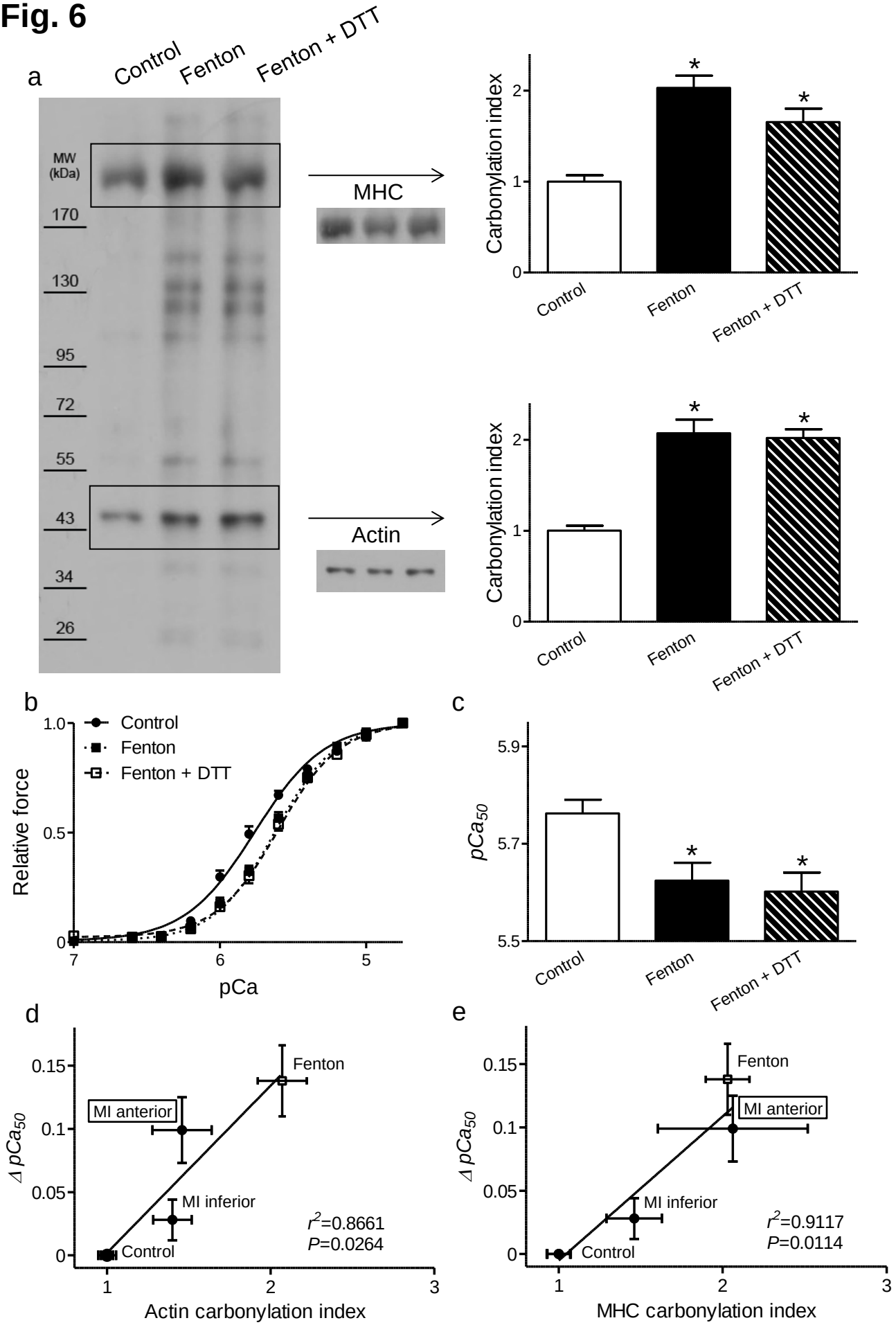
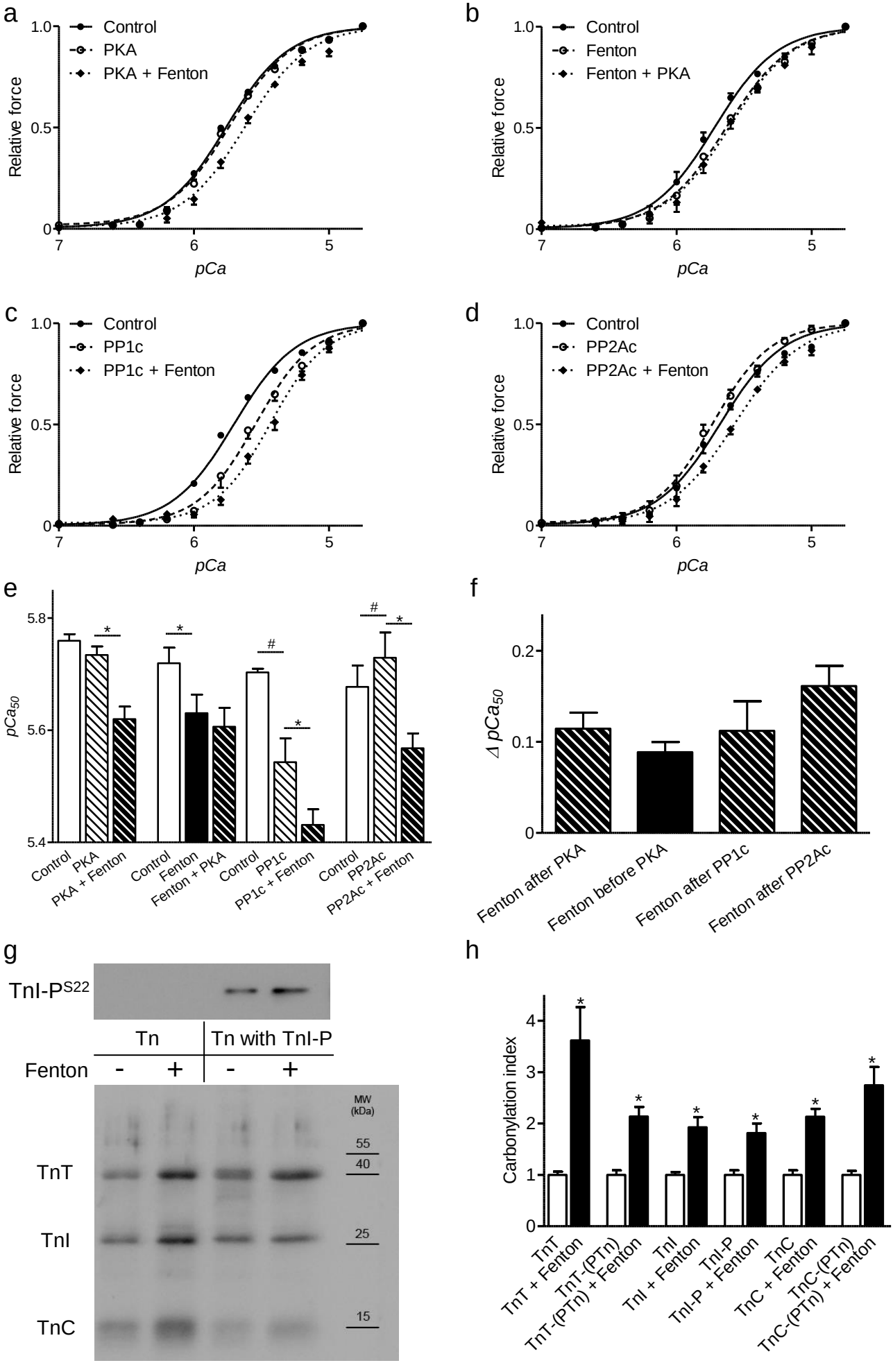

a
Control
Fenton
Fenton + DTT
MW (kDa)
170
130
95
72
55
43
34
26
MHC
Actin
Carbonylation index
Control
Fenton
Fenton + DTT
b
Control
Fenton
Fenton + DTT
Relative force
pCa
c
pCa50
d
MI anterior
MI inferior
Fenton
Control
r2=0.8661
P=0.0264
Δ pCa50
Actin carbonylation index
e
MI anterior
MI inferior
Fenton
Control
r2=0.9117
P=0.0114
Δ pCa50
MHC carbonylation index

Fig. 7


a
Control
PKA
PKA + Fenton
Relative force
pCa
b
Control
Fenton
Fenton + PKA
Relative force
pCa
c
Control
PP1c
PP1c + Fenton
Relative force
pCa
d
Control
PP2Ac
PP2Ac + Fenton
Relative force
pCa
e
pCa50
Control
PKA
PKA + Fenton
Control
Fenton
Fenton + PKA
Control
PP1c
PP1c + Fenton
Control
PP2Ac
PP2Ac + Fenton
f
Δ pCa50
Fenton after PKA
Fenton before PKA
Fenton after PP1c
Fenton after PP2Ac
g
TnI-PS22
Tn
Tn with TnI-P
Fenton
MW (kDa)
55
40
25
15
TnT
TnI
TnC
h
Carbonylation index
TnT
TnT + Fenton
TnT-(PTn)
TnT-(PTn) + Fenton
TnI
TnI + Fenton
TnI-P
TnI-P + Fenton
TnC
TnC + Fenton
TnC-(PTn)
TnC-(PTn) + Fenton

Supplementary material

Survival data

10 of 25 LAD-ligated animals survived the 10-week follow-up period, i.e. an overall postoperative survival rate of 40%. The early operative mortality (<48 h) in the MI group was 44% (11/25), and a further 4/25 (16%) mice died between 48 h and one week after ligation. All the control animals survived the 10-week follow-up period.

Infarct size determination

Under anesthesia (100 mg/kg ketamine and 12 mg/kg xylazine, i.p.), beating hearts were excised and washed in cold (4 °C) Krebs-Henseleit solution with a composition (concentrations in mM) of NaCl: 118, KCl: 4.7, $CaCl_2$: 2.5, $MgSO_4$: 1.2, K_2HPO_4: 1.2, ethylenediaminetetraacetic acid (EDTA): 0.5, $NaHCO_3$: 25, glucose: 11.1 (Merck, Darmstadt, Germany). Next, the hearts were either fixed in 4% formaldehyde for histologic analyses, or were dissected for isometric force measurements or for molecular assays according to the following protocol: first the atria and the great vessels, and then the right ventricle were separated from the LV, and finally the infarcted area was separated from the noninfarcted, inferior segment of the LV. Infarction size was determined as described previously.[1] Briefly, formalin-fixed LVs were embedded in paraffin and sectioned in 4-µm slices with a microtome. For the acquisition of pertinent measurements from the whole LV, five transversal sections from base to apex were stained with Masson's trichrome. Subsequently, endo- and epicardial LV circumferences from all sections were quantified with Image J software (Image J, National Institute of Health, USA) and summed to obtain the total LV circumference. For the infarction size, the total infarct length of all five sections was divided by the total LV circumference.

Force measurements in permeabilized cardiomyocyte preparations

The technique for isolation of the cardiomyocyte preparations, and the compositions of the activating and relaxing solutions used during the mechanical measurements, have been described in detail elsewhere.[2] Briefly, after mechanical isolation, 0.5% Triton-X 100 detergent (in relaxing solution) was used for permeabilization. The *pCa*, i.e. $-\log_{10}[Ca^{2+}]$, of the relaxing solution was 9, while the activating solution had a *pCa* of 4.75. Both solutions contained protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 40 μM leupeptin and 10 μM E-64 (all chemicals from Sigma-Aldrich, St. Louis, MO, USA).

With the use of silicone adhesive (Dow Corning 100% silicone aquarium sealant; Midland, MI, USA), a single cardiomyocyte was attached to two thin stainless steel needles, which were connected to a force transducer (SensoNor, Horten, Norway) and to an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada). Isometric force measurements were performed during repeated activation–relaxation cycles at 15 °C, first at a sarcomere length (SL) of 1.9 μm and then at a SL of 2.3 μm. Ca^{2+} contractures were evoked by transferring the cardiomyocyte from Ca^{2+}-free relaxing solution to activating solutions of gradually decreasing $[Ca^{2+}]$. During single Ca^{2+} contractures, when the force reached the maximal value, a quick release-restretch maneuver was performed in the activating solution and the rate constant of force redevelopment ($k_{tr,max}$) was determined. As a result of this intervention, the force dropped from peak level to zero, allowing determination of the total force level (F_{total}), and then started to redevelop. The cardiomyocyte was then returned to the relaxing solution, where a shortening of the original preparation length to 80% was performed to assess the passive force level ($F_{passive}$). The active isometric force (F_o) was calculated by subtracting $F_{passive}$ from F_{total}. F_o and $F_{passive}$ were normalized for the cardiomyocyte cross-sectional area calculated from the width and height of the cardiomyocytes. Isometric force values were normalized for the maximal Ca^{2+}-activated active force, and Ca^{2+}–force relations were plotted to determine the Ca^{2+} sensitivity of isometric force production, i.e. pCa_{50}.

During our experimental protocol, the effects of incubations in the presence of the catalytic subunit of bovine heart PKA (relative phosphorylating effect of ~10 U/μg; Sigma-Aldrich, St. Louis, MO, USA) on pCa_{50} were also assessed. To this end, 1 mg of lyophilized PKA was added to 60 μl of 100 mM dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO, USA). This mixture was diluted to 1 ml with relaxing solution, and stored at 4 °C for no more than 5 days prior to use. To assess the role of sulfhydryl (SH) oxidation on the mechanical parameters, cardiomyocytes were exposed to antioxidant treatment (10 mM DTT in relaxing solution).

Isometric force measurements were conducted at a SL of 2.3 μm in response to *in vitro* PKA or DTT treatment. After assessment of the initial Ca^{2+}–force relationship, the preparation was incubated in the PKA mixture for 40 min or in DTT solution for 30 min, and the Ca^{2+}–force relationships were then determined again.

Investigation of the phosphorylation status of cardiac TnI

A modified RIPA (radio-immunoprecipitation assay) solution (pH=8, Tris-HCl: 50 mM, NaCl: 150 mM, Igepal: 1%, sodium deoxycholate: 0.5%, sodium dodecylsulfate (SDS): 6%, protease inhibitor cocktail: 1%; all components from Sigma-Aldrich, St. Louis, MO, USA) was used for the preparation of protein lysates. The homogenates were mixed with equal volumes of 2x SDS sample buffer (Sigma-Aldrich, St. Louis, MO, USA) and were boiled for 10 min. After electrophoresis and blotting, nitrocellulose membranes were probed with antibodies against S22/S23- or T143-phosphorylated cardiac TnI (Abcam, Cambridge, UK; anti-$P^{S22/S23}$-TnI, dilution: 1:1000 in phosphate-buffered saline containing 0.1% Tween-20 (PBST); anti-P^{T143}-TnI, dilution: 1:500 in PBST). The signals were detected by horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Millipore, Billerica, MA, USA; dilution: 1:300 in PBST). After stripping, membranes were probed with phosphorylation-insensitive antibodies against cardiac TnI (Clone 19C7; Hytest Ltd., Finland; dilution: 1:10,000 in PBST) and peroxidase-conjugated (POD) anti-mouse secondary antibodies (Sigma-Aldrich, St.

Louis, MO, USA; dilution: 1:20,000 in PBST). The bands were visualized by an enhanced chemiluminescence (ECL) method and autoradiography, and evaluated with Image J software for both phosphorylated and total cardiac TnI. Relative phosphorylation was calculated after a normalization step.

Detection of protein carbonyl groups

Protein lysates for oxyblot assays were prepared with the help of RIPA solution as above. Protein concentrations were determined by a bicinchoninic acid (BCA) assay, using bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO, USA) as standard, and the concentrations of the homogenates were adjusted to 3 mg/ml. All further steps were performed on the basis of the kit brochure. Briefly, the carbonyl groups of the protein side-chains were derivatized to 2,4-dinitrophenylhydrazone (DNPhydrazone) by reacting with 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis (PAGE), followed by Western immunoblotting. The membranes were probed with antibodies derived from the kit diluted in PBST containing 1% BSA. After stripping, anti-actin (DAKO A/S, Glostrup, Denmark; dilution: 1:1000 in PBST), anti-α-actinin (Sigma Aldrich, St. Louis, MO, USA; dilution: 1:10,000 in PBST) and anti-myosin heavy chain (MHC) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA; dilution: 1:10,000 in PBST) antibodies were employed to estimate the total amounts of these proteins. Protein bands were visualized by the ECL method and autoradiography, and evaluated with Image J software. Relative protein carbonylation values, i.e. carbonylation indices (*CI*) were calculated with normalization for protein amounts.

***In vitro* protein carbonylation**

Fenton treatment (50 µM $FeSO_4$, 1.5 mM H_2O_2, 6 mM ascorbic acid in relaxing solution for 7 min at room temperature) was performed on isolated, permeabilized control cardiomyocytes, either in the mechanical set-up or before oxyblot assays in test-tubes. DTT (10 mM) was used

to reduce oxidized SH groups. Thereafter, cardiomyocytes were suspended in RIPA buffer, and an oxyblot assay was performed as described previously. The mechanical characteristics of the permeabilized cardiomyocytes were evaluated by direct force measurements before and after exposure of the cardiomyocytes to the Fenton reaction and after subsequent DTT treatment (10 mM, 30 min), additionally before and after PKA or after PP1c or PP2Ac treatments. The presence of Fenton-induced SH oxidation was tested by Ellman's reaction. SH content was determined by incubation of the permeabilized treated and untreated cardiomyocytes with the SH-sensitive Ellman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid); Sigma-Aldrich, St. Louis, MO, USA] for 15 min at room temperature. The absorbance of the solutions was measured at 412 nm by using a NOVOstar Microplate Reader and was considered to be proportional to their SH contents. The samples were assessed via calibration curves (standard: *N*-acetyl-L-cysteine; Sigma-Aldrich, St. Louis, MO, USA) fitted to a single exponential, and the SH contents of the myocardial samples were calculated for 1 mg protein. Protein concentration was assessed by using bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) as a standard.

Investigation of SH oxidation

A biotin-streptavidin system was employed for the evaluation of protein SH groups.[3, 4] A solution (pH=8.3) containing 50 mM Tris-HCl and 5 mM EDTA was used for biotinylation, with desalt spin columns (Zeba Micro Desalt Spin Columns, Zeba Spin Desalting Columns; Pierce Biotechnology, Rockford, IL, USA) for buffer exchange. After *in vitro* incubations either in DTT or in relaxing solution, DTT was removed with the same columns as used for buffer exchange. Protein homogenates were incubated with biotin [(+)-biotinyliodoacetamidyl-3,6-dioxaoctanediamine; Pierce Biotechnology, Rockford, IL, USA], dissolved in dimethylformamide for 90 min in the dark at 37 °C, and then mixed with an equal volume of 2x SDS sample buffer (Sigma-Aldrich, St. Louis, MO, USA) and boiled for

10 min. After PAGE and blotting, a streptavidin-peroxidase system (Jackson ImmunoResearch Europe Ltd., Suffolk, UK; dilution: 1:100,000 in PBST) was used for signal detection. After stripping of the membranes, anti-actin antibodies (DAKO A/S, Glostrup, Denmark; dilution: 1:1000 in PBST) and anti-mouse POD (Sigma-Aldrich, St. Louis, MO, USA; dilution: 1:20,000 in PBST) secondary antibodies were used to determine the total amount of actin. The bands were visualized by the ECL method and evaluated with Image J software for both SH groups and actin signals. Finally relative SH oxidation was calculated.

Carbonylation of recombinant troponin complexes

Recombinant troponin complexes were prepared by Jaquet's group as described earlier.[5] After derivatization and blotting, Sypro Ruby protein blot stain was used to establish the protein amount (Life Technologies, Carlsbad, CA, USA). Oxyblot was performed to determine carbonylation levels. TnI phosphorylation status was confirmed with phosphorylation-sensitive antibody. The bands were visualized by the ECL method and evaluated by Image J software (free software from National Institutes of Health, Bethesda, MD, USA). Band intensities were normalized for the amount of each protein visualized by Sypro Ruby stain. Assays were performed twice in triplicates. Data are shown in carbonylation indices (*CI*) as above.

Data analysis, statistics

The Ca^{2+}–force relations were fitted to a modified Hill equation:

$$F = F_o[Ca^{2+}]^{nHill}/(Ca_{50}{}^{nHill} + [Ca^{2+}]^{nHill})$$

where F is the steady-state force at a given $[Ca^{2+}]$, while F_o, $nHill$ and Ca_{50} (or pCa_{50}) denote the maximal Ca^{2+}-activated force at saturating $[Ca^{2+}]$, and the slope and the midpoint of the sigmoidal relationship, respectively. The force redevelopment after the release–restretch maneuver was fitted to a single exponential function in order to estimate the rate constant of force redevelopment ($k_{tr,max}$) at the maximal $[Ca^{2+}]$ level:

$$F(t) = F_i + F_a\,(1 - e^{-k_{tr,max}t})$$

where $F(t)$ is the force at any time (t) after the restretch at the maximal [Ca^{2+}], and F_i and F_a denote the initial force after the restretch and the amplitude of Ca^{2+}-activated force redevelopment, respectively. Each experimental preparation was fitted individually, the fitted parameters were pooled, and the mean values are reported.

Figure legend

Suppl. Fig. 1 The cross-sectional area showed no significant differences between the cardiomyocytes derived from the different experimental groups. Cross-sectional area was similar in Control, MI anterior and inferior locations at sarcomere lengths of both 1.9 and 2.3 μm.

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