Oxidative modification of tropomyosin and myocardial dysfunction following coronary microembolization

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Aims We addressed a potential mechanism of myocardial dysfunction following coronary microembolization at the level of myofibrillar proteins.

Methods and results Anaesthetized pigs underwent intracoronary infusion of microspheres. After 6 h, the microembolized areas (MEA) had decreased systolic wall thickening to 38 ± 7% of baseline and a 2.62 ± 0.4-fold increase in the formation of disulphide cross-bridges (DCB) in tropomyosin relative to that in remote areas. The impairment in contractile function correlated inversely with DCB formation (r = −0.68; P = 0.015) and was associated with increased TNF-α content. DCB formation was reflected by increased tropomyosin immunoreactivity and abolished in vitro by dithiothreitol. Ascorbic acid prevented contractile dysfunction as well as increased DCB and TNF-α. In anaesthetized dogs, 8 h after intracoronary microspheres infusion, contractile function was reduced to 8 ± 10% of baseline and DCB in MEA was 1.48 ± 0.12 higher than that in remote areas. In conscious dogs, 6 days after intracoronary microspheres infusion, myocardial function had returned to baseline and DCB was no longer different between remote and MEA. Again contractile function correlated inversely with DCB formation (r = −0.83; P = 0.005).

Conclusion Myofibrillar protein oxidation may represent a mechanistic link between inflammation and contractile dysfunction following coronary microembolization.

Introduction

Plaque rupture in an atherosclerotic coronary artery does not necessarily result in complete coronary occlusion and impending transmural myocardial infarction. The plaque debris may rather be embolized into the coronary microcirculation, resulting in arrhythmias, inflammation, progressive contractile dysfunction, and microinfarcts.1 We have recently characterized an experimental model of coronary microembolization in dogs and pigs, where reversible contractile dysfunction is induced through a signal cascade involving TNF-α and sphingosine.2–4 We have now addressed a potential mechanistic link between the inflammatory signal transduction and the contractile impairment. Reactive oxygen species (ROS) have a dose-dependent, ambivalent action in the heart. Small amounts of ROS may serve a signalling function and result in cardioprotection,5–8 whereas larger amounts result in myocardial damage.9 In the scenario of myocardial ischaemia/reperfusion, small amounts of ROS are important signalling elements in ischaemic pre-conditioning,6,8 whereas large amounts of ROS cause stunning10 and cell death.11

Oxidation can affect the side chains of most amino acid residues.12 Carbonylation mostly affects lysine, arginine, and proline and is irreversible. In contrast, the formation of intra- and intermolecular disulphides is reversible and can be enzymatically repaired.13 Both reversible and irreversible oxidative modifications of cardiac myofibrillar proteins have been reported during oxidative stress and myocardial ischaemia/reperfusion.14–20 We have recently identified tropomyosin as a target of reversible oxidation.20 Although our prior findings are consistent with the hypothesis that the reversible contractile impairment in stunning is related to covalent changes of myofibrillar proteins, these studies originate from isolated saline-perfused rodent hearts, where myocyte death inevitably contributes to loss of function, and a correlation of myofibrillar protein oxidation to contractile function is not available.

We therefore used our established dog and pig in situ models of coronary microembolization to study the relations between the reversible contractile dysfunction, the inflammatory cytokine TNF-α and the oxidative modification of myofibrillar proteins as a potential mechanistic link. To support a role of oxidative myofibrillar protein modification in contractile dysfunction, we used ascorbic acid as an antioxidant, which we have previously demonstrated by electron spin resonance to scavenge ROS and eliminate...
their signalling function for ischaemic pre-conditioning in the same preparation. 8

Methods
Experimental preparation and protocols
In anaesthetized pigs (Göttingen Minipig, Ellegard, Dalmore, Denmark; body weight 35–40 kg), the left anterior descending coronary artery was perfused from a carotid artery with an occlusive roller pump, and microspheres (3000 per mL/min coronary inflow; 42 μm diameter) were injected into the perfusion system. 4 Regional systolic wall thickening was measured using sonomicrometry. After 6 h, the pigs were euthanized and tissue taken for subsequent analysis. In detail, the heart was excised and immediately cut into five slices perpendiculard to the LV long axis. Samples of ~1 g from the three central slices were cut, both from the central microembolized area and a remote control area. The samples were immediately frozen and stored in liquid nitrogen. For the analysis of the myocardial TNF-α concentration, about 200 mg of tissue from the microembolized area and the remote area were stored at −70 °C and homogenized in a microdismembranator (B. Braun, Melsungen, Germany) in cold isonicotinic homogenization buffer (in mmol/L: imidazol acetate 50, Mg acetate 10, and KH2PO4 4, in addition to 2 mmol/L EDTA; in μmol/L: N-acetylcysteyne 50, sulphur 12.5; pH 7.6). Samples were centrifuged at 2000 g for 15 min at 4 °C and supernatants collected.

TNF-α concentration was measured using a cytolytic cell assay (mouse fibrosarcoma cell line WEHI 164, Clone 13), using recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA) as standard. 2

Nine pigs received physiological saline (placebo). In a second step, four additional pigs received ascorbic acid (i.v. bolus 2 g/animal followed by i.v. 25 mg/min maintenance dose according to Skyschally et al.).8 The intravenous ascorbic acid regimen has previously been demonstrated to scavenge ROS by electron spin resonance and to eliminate their signalling function in ischaemic pre-conditioning. 8 In four anaesthetized dogs, the microspheres were injected through a 25 gauge cannula into the left circumflex coronary artery and the experiments were terminated after 8 h. 2 To follow eventual recovery of contractile function, in five additional chronically instrumented dogs, the microspheres were injected under light anaesthesia and fluoroscopic control through a catheter introduced from a femoral artery, and the experiments were terminated after 6 days. 3 In both, the pig and the dog models, the aggregate size of patchy microinfarcts is <5% of the microembolized myocardium, 3,4 and it is not altered by cortisone 3 in dogs (1.8 ± 1.9% after 8 h and 2.2 ± 0.8% after 6 days) or ascorbic acid in pigs (2.9 ± 2.2 vs. 2.7 ± 1.9% in placebo, present study). Apoptosis is minimal in this model. 21

Protein extraction and immunoelectrophoresis
Tropomyosin was assessed in a blinded manner by standard western blot analysis. Briefly, heart biopsies were stored in liquid nitrogen (~20 mg) and then homogenized in ice-cold PBS, pH 7.2 containing an antiprotease mixture (10 μg leupeptin, 10 μg pepstatin, and 0.2 mm phenylmethylsulphonyl fluoride) and 5 mm EDTA. Just before use, the solution was stirred under vacuum and bubbled with 10% CO2. To reduce the protein suspension was centrifuged at 12 000 g for 10 min at 4 °C. The resulting pellet was resuspended in sample buffer (2% SDS, 5% glycerol, 1% β-mercaptoethanol, 125 mm Tris–HCl, pH 6.8) and denatured by 10 min boiling. This procedure referred to as reducing condition was compared with the non-reducing condition obtained without β-mercaptoethanol. To avoid artefacts due to the oxidation of thiol groups in vitro, non-reducing conditions were performed in the presence of 1 mm N-ethylmaleimide. Tropomyosin oxidation was also detected in the supernatant, and the ratio of tropomyosin oxidation in the microembolized to that in the remote sample was similar to that in the pellet.

SDS–PAGE was performed in mini gel format (7 cm gel size) using the BioRad Protein II Electrophoresis system (Bio-Rad, Hercules, CA, USA). Protein samples (12 μg) were loaded on 12% SDS–PAGE separating gels and then transferred to 0.45 μm pore-size nitrocellulose membranes (Bio-Rad) at 150 mA constant current for 16 h in a buffer containing 25 mm Tris–HCl, 192 mm glycine, and 20% methanol. 22 The efficiency of transfer to nitrocellulose membrane was checked by staining with Red Ponceau S (Sigma Chemical Co., St. Louis, MO, USA). The membranes were blocked by incubation with 3% BSA for 1 h and then incubated for 2 h at room temperature with an anti-tropomyosin monoclonal antibody (CH1 clone, Sigma Chemical Co.) and revealed by anti-mouse immunoglobulin conjugated with horseradish peroxidase (Dako, Glostrup, Denmark). Signals were visualized by a chemiluminescence detection system checking that exposures were within the linear range of detection. Densitometry was performed on scanned immunoblots using the IPLab Gel computer program for the Macintosh (Signal Analytics Co., Fairfax, VA, USA).

Quantitative analysis of the degree of tropomyosin oxidation was performed on the densitometric values of the bands detected in immunoblots. In particular, the density of the additional band with higher molecular weight reflecting the formation of disulphide cross-bonds (DCB) was normalized to Red Ponceau staining to take differences in sample loading into account. Each western blot analysis was carried out by loading samples from the microembolized and non-microembolized area of the same heart.

To investigate the occurrence of protein oxidation of other myofilament proteins at the level of cysteine residues, after electrophoretic separation of samples electrophoresed under reducing and non-reducing conditions the membranes were probed with the following monoclonal antibodies: (i) anti-αs sarcomeric actin 5C5 clone (Sigma Chemical Co.), (ii) anti-TnC (Mab Bi-7, Spectral diagnostic, Toronto, Ontario, Canada), (iii) anti-TnC (Biodesign, Saco, ME, USA), and (iv) anti-desmin DE-B-5 clone (Oncogene, Cambridge, MA, USA). TnT was not tested because it does not contain cysteine residues.

To verify the identity of the second band as an oxidized tropomyosin product, two approaches were used. The blots from microembolized myocardium were probed with the antibodies mentioned above. In addition, purified human cardiac tropomyosin (Abcam, Cambridge, UK) was oxidized, as originally reported. 22 Briefly, alpha-tropomyosin (4 μg) was incubated with 2 mm 5,5′-dithiobis(2-nitrobenzoate) in 1 m NaCl, 1 mM EDTA, 0.05 m Na phosphate, pH 7.4 for 1 h at room temperature and then loaded in non-reducing SDS–PAGE.

Immunohistochemistry
Cryosections were incubated with anti-tropomyosin monoclonal antibodies (CH1 clone) for 30 min (1:25 in PBS containing 0.3% BSA). After several rinses with PBS, sections were incubated with fluorescein-conjugated secondary antibodies (Dako). The fluorescence images were acquired with an Olympus IMT-2 inverted microscope, equipped with a xenon lamp and a 12-bit digital cooled CCD camera (Micromax, Princeton Instruments, Monmouth Junction, NJ, USA) as previously described. 23 For detection of the fluorescein fluorescence, 488 ± 25 nm excitation and 522 nm long-pass emission filter settings were used. Data were acquired and analysed using Metamorph software (Universal Imaging, West Chester, PA, USA).

To verify the relationship between tropomyosin oxidation and immunoreactive changes, cryosections from the remote area were incubated for 30 min with an oxidizing solution containing 0.5 mm H2O2 and 0.2 mm FeSO4 in PBS. After several rinses with PBS, the cryosections were incubated with the anti-tropomyosin antibody (clone CH1) as described above. In parallel, cryosections from the microembolized area(s) were incubated 30 min with PBS containing

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5 mM dithiothreitol, washed with PBS, and subsequently probed with the anti-tropomyosin antibody.

Data analysis and statistics
Mean values ± SEM are reported. DCB ratios of microembolized to remote myocardium were compared between placebo and ascorbic acid in pigs and between 8 h and 6 days in dogs by unpaired, two-sided t-tests. The functional data in pigs with placebo and ascorbic acid over time were compared by two-way ANOVA, and the immunoreactivities in microembolized and remote myocardium without and with ascorbic acid were compared by two-way ANOVA and post hoc tests (Fisher LSD, Bonferroni correction for multiple testing). Linear regression analyses were performed to analyse the quantitative relation between myocardial dysfunction and the amount of the oxidative tropomyosin modification, as indicated by DCB formation. A P-value less than 0.05 was taken to indicate a significant difference.

Results
As shown by the typical example in Figure 1, immunoblots stained with the tropomyosin monoclonal antibody CH1 clone displayed an additional band (apparent molecular weight of 82 kDa) in biopsy samples obtained from the microembolized areas (MEA) of pig hearts 6 h after microembolization. As the appearance of high molecular weight bands could also result from other cross-linking processes, such as the activation of transglutaminase, the attribution to DCB formation is performed by comparing the immunoblots obtained after SDS–PAGE carried out under reducing and non-reducing conditions. The appearance of this band, which was quite faint in samples obtained from non-embolized areas (RA), reflected DCB formation, because it was visible only in non-reducing electrophoresis. Also, this signal was specific for tropomyosin oxidation, as it did not stain positive for troponin I or C (data not shown) and as the same band appeared with induced oxidation of purified tropomyosin (Figure 1). As shown in Figure 2, besides affecting contractility as previously described, microembolization resulted in higher DCB amounts in microembolized than in remote, non-embolized areas. Indeed, the ratio between the densities of DCB in samples from MEA and RA was 2.62 ± 0.40. This ratio was reduced to 1.05 ± 0.21, indicating no difference between MEA and RA samples, when microembolization was preceded by the intravenous infusion of ascorbic acid, a treatment shown to effectively scavenge ROS in this same experimental model.

The formation of cross-linked products is likely to modify protein conformation, possibly resulting in changes of immunoreactivity. Indeed, the occurrence of such a process has been demonstrated for another myofibrillar protein, troponin T, which undergoes transglutaminase-catalysed cross-linking upon post-ischaemic reperfusion. Therefore, we analysed tropomyosin immunoreactivity in cryosections from pig hearts subjected to microembolization. Figure 3 shows that tropomyosin immunoreactivity was homogeneously increased in microembolized cryosections with respect to remote controls. The quantitative analysis indicates a significant increase of the immunofluorescence values.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Detection of DCB in tropomyosin induced by coronary microembolization. Myofibrillar proteins were extracted from pig heart biopsies obtained 6 h after coronary microembolization. MEA, samples from microembolized areas; RA, samples from non-microembolized, remote areas. Isolated myofibrillar samples were denatured in the absence (non-reducing conditions) or in the presence (reducing conditions) of β-mercaptoethanol and then analysed by western blot probed with CH1 anti-tropomyosin monoclonal antibodies. High molecular weight bands were detected by western blot after microembolization and represent DCB, because they were present only in non-reducing conditions. Oxidation of purified human tropomyosin (Tm ox) results in a band with similar electrophoretic mobility of that observed in microembolized samples. Bottom: Red Ponceau staining to reflect protein loading.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Microembolization-induced contractile dysfunction and tropomyosin oxidation are attenuated by ascorbic acid. Pigs were treated with placebo (saline) or ascorbic acid. Systolic wall thickening was decreased by coronary microembolization and quickly recovered to a similar extent in placebo (closed circle) and ascorbic acid-treated (open square) pigs. A secondary slowly progressive dysfunction developed in placebo pigs and was largely attenuated in ascorbic acid-treated pigs. Insert: the extent of tropomyosin oxidation was quantitatively evaluated as the increase in the density of the high molecular weight band, resulting from DCB formation. The increase was expressed as the ratio (DCB ratio) between the densities in MEA samples and RA samples. Ascorbic acid largely attenuated the increase in DCB ratio.
Other processes activated by microembolization could have contributed to the immunofluorescence changes, especially covalent changes of myofibrillar proteins, such as phosphorylation and proteolysis. Figure 4 illustrates the experiments performed to validate the attribution of the increase in immunofluorescence to oxidative stress. Tropomyosin immunofluorescence increased when cryosections from control untreated samples (i.e. remote areas) were added (in vitro) with an oxidizing solution containing 0.5 mM H2O2 and 0.2 mM FeSO4. In contrast, the addition of dithiothreitol, a reducing agent, to cryosections from MEA completely abolished the increase in immunofluorescence, allowing the non-ambiguous attribution of the change in tropomyosin immunoreactivity to its oxidation.

Confirming its antioxidant efficacy previously documented in this same experimental model,8 the increase in tropomyosin immunoreactivity induced by microembolization was significantly reduced by ascorbic acid (Figure 3). It could be argued that this effect might be the consequence of a non-specific protection afforded by the antioxidant treatment rather than a direct effect of radical scavenging on myofibrillar protein oxidation. However, ascorbic acid was equally effective in decreasing DCB formation (Figure 2), thus documenting its direct effect on tropomyosin oxidation.

Ascorbic acid largely attenuated also the contractile dysfunction (Figure 2), and contractile function was inversely correlated to the DCB ratio (Figure 5). In addition, the decrease in tropomyosin oxidation was accompanied by a reduced accumulation of TNF-α (Figure 6), suggesting that myofibrillar protein oxidation may be a final step in the sequence of events from inflammation to contractile failure.

In anaesthetized dogs, there was severe dysfunction 8 h after coronary microembolization (systolic wall thickening 8 ± 10% of baseline) and increased DCB ratio (1.48 ± 0.12). Contractile function recovered completely back to baseline after 6 days (105 ± 10% of baseline) and also DCB density was no longer different in microembolized and remote myocardium (DCB ratio: 1.03 ± 0.04, P = 0.006 vs. 8 h). The DCB ratio correlated inversely to contractile function (Figure 5).
Discussion

The present results demonstrate that tropomyosin is oxidized in pig and dog hearts upon coronary microembolization. The extent of tropomyosin oxidation correlates inversely with contractile function, establishing for the first time a quantitative relationship between myofibrillar protein oxidation and contractile impairment. In addition, TNF-α content is increased in parallel with tropomyosin oxidation. Therefore, the oxidation of contractile proteins might represent an end-effector of the transduction pathway triggered by microembolization that links the inflammatory response to the failure of contraction.

The occurrence of myofibrillar protein oxidation has previously been reported in isolated rodent hearts perfused at supraphysiological P O₂ subjected to oxidative stress or ischaemia/reperfusion protocols. In such preparations, myocyte death inevitably contributes to loss of contractile function, and myofibrillar protein oxidation was never correlated with contractile derangements directly. In the present study, irreversible myocyte loss was <5% of the microembolized myocardium, and this myocyte loss was not affected by ascorbic acid.

We chose to investigate DCB formation in tropomyosin because preliminary experiments carried out in rat hearts showed that this process was associated with a modification of immunoreactivity detectable by using the tropomyosin monoclonal antibody CH1 clone. This finding is now validated in microembolized myocardial samples, also according to the previous studies which linked protein oxidation to immunoreactive changes.

It must be pointed out that the present study provides the first immunohistochemistry evidence of protein oxidation, which previously has been shown only by means of immunoblotting analyses. We found oxidation also of actin and desmin in a few samples. We only thoroughly investigated tropomyosin and its oxidation, which also went along with modification of its immunoreactivity. No oxidation was detected in troponin C and I.

We investigated the quantitative relationships between loss of function and myofibrillar protein oxidation by using the non-ambiguous evidence of tropomyosin oxidation provided by immunoblots. To this aim, we developed a quantitative analysis of tropomyosin oxidation based on DCB formation. A significant increase in the extent of tropomyosin oxidation was detected in samples obtained 6 h after microembolization when the contractile function is maximally depressed. In dog hearts, complete recovery of function occurred after 6 days and was associated with an almost complete disappearance of DCB whose amounts were no longer different from those detected in non-microembolized, 

Figure 5 Tropomyosin oxidation correlates with contractile dysfunction—relationship between the ratio of MEA and RA samples and systolic wall thickening (per cent of baseline). (A) Pig hearts 6 h after coronary microembolization (open square, placebo; filled square ascorbic acid). Of note, one placebo point is missing due to technical problems with sonomicrometry. (B) Dog hearts 8 h after coronary microembolization (open square) and 6 days after coronary microembolization (filled square).

Figure 6 The microembolization-induced increase of myocardial TNF-α concentration is prevented by ascorbic acid. TNF-α contents were assayed in samples from microembolized (filled bars, MEA) and remote (open bars, RA) areas.
remote areas. The differences between samples from microembolized and control areas were not observed in pigs treated with ascorbic acid that was also efficacious in preventing the contractile abnormalities.

Given the currently available methodology, our study cannot exclude oxidation or other post-translational modifications of myofibrillar proteins which may be critical for the development of contractile dysfunction. Also, alterations in calcium homeostasis will undoubtedly contribute to contractile dysfunction. However, it must be pointed out that in most cases the hypotheses suggesting the contribution of the oxidation of a specific protein have not been substantiated by qualitative and quantitative analysis of the occurrence of protein oxidation in situ. The present study is the first to quantify the extent of protein oxidation in situ establishing a correlation with mechanical dysfunction.

The amount of tropomyosin undergoing DCB formation appears rather modest suggesting that this process is not the only one responsible for contractile dysfunction. Indeed, it is likely that the alteration of contractile function results from the sum of several oxidative processes affecting various proteins. The present results suggest that the extent of protein oxidation in relation to contractile dysfunction appears to be indicated reliably by tropomyosin. Nevertheless, tropomyosin oxidation might play a role beyond that of simply probing the oxidation of myofibrillar proteins. This hypothesis is supported by the site of tropomyosin oxidation. In fact, the only cysteine residue of cardiac tropomyosin (Cys190) is located at the interface with troponin T. DCB formation causing large conformational modifications, as shown by the present evidence of immunofluorescence changes, is likely to alter protein interactions that are crucial for contractile activity. This concept is supported by experimental models and clinical findings relating tropomyosin alterations to contractile abnormalities. Regarding the relatively modest amount of tropomyosin oxidized, it has to be pointed out that severe contractile abnormalities have been reported for cardiomyocytes harbouring a minor fraction of altered myofibrillar proteins. Indeed, this was the case with the original report of inborn error of tropomyosin.

The present results demonstrate that tropomyosin oxidation parallels the increase in TNF-α content, confirming and extending previous observations linking oxidative stress and inflammation with contractile impairment. Of note, previous reports lack both quantitative information and identification of molecular target(s) that are provided by the present study. Similarly, the beneficial effect of antioxidant treatments documented by many studies does not appear to be a plausible mechanism. The decrease of TNF-α induced by ascorbic acid confirms recent reports demonstrating that in various cell types TNF-α synthesis depends on ROS formation. Vice versa, TNF-α promotes ROS formation, thus establishing a bidirectional link that is likely to amplify the inflammatory response by exacerbating the oxidative stress. In fact, our results indicate that ascorbic acid treatment prevents both TNF-α accumulation and tropomyosin oxidation and suggest that myofibrillar protein oxidation is a potential mechanism of the TNF-α-induced decrease in contractile function described in previous reports.

The present results cannot unequivocally identify the mechanism(s) which underlie(s) the reversibility of the contractile failure induced by microembolization. DCB formation is a reversible process and thiol–disulfide transitions modulate protein tyrosine phosphatase activity. On the other hand, oxidized proteins have been suggested to be preferentially degraded by intracellular proteolytic systems. The time required for the complete recovery of contractile performance would suggest that the prevailing mechanism is de novo synthesis of damaged proteins rather than enzymatic repair. Interestingly, the recovery time of 6 days found in dogs with microembolization is similar, but somewhat longer than that observed in myocardial stunning and is quite similar to the reported half-time for the synthesis of troponin and tropomyosin.

In conclusion, tropomyosin is oxidized with coronary microembolization, its conformational change is likely to affect contractile function, and this tropomyosin oxidation may well causally contribute to the observed contractile dysfunction.

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