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Myofilament Carbonylation Modulates Contractility in Human Cardiomyocytes

Original article

Ágnes Balogh, Attila Tóth, Enikő Tóth Pásztorné, László Nagy, Árpád Kovács, Judit Kalász, Gerardo Alvarado Contreras, István Édes and Zoltán Papp*

Division of Clinical Physiology, Institute of Cardiology, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary

*Correspondence: Zoltán Papp, M.D., Ph.D., D.Sc., Division of Clinical Physiology, Institute of Cardiology, University of Debrecen, Medical and Health Science Center, Móricz Zs. krt. 22, H-4032 Debrecen, Hungary, Tel./Fax: +36-52-255928, E-mail: pappz@med.unideb.hu

Abstract This study investigated the effects of myofilament carbonylation in permeabilized human left ventricular cardiomyocytes. Protein carbonylation was monitored by the oxyblot method following the *in vitro* application of the Fenton reaction reagents [iron(II), ascorbic acid and hydrogen peroxide (H_2O_2)], known to produce hydroxyl radicals. Sulfhydryl group oxidation was assessed in parallel by the Ellman assay. During force measurements, the Ca^{2+}-activated active force, the Ca^{2+}-independent passive force and the Ca^{2+} sensitivity of force production (pCa_{50}) were measured in permeabilized cardiomyocytes before and after *in vitro* carbonylation. Carbonylation at the levels of several myofilament proteins (*e.g.* myosin heavy chain, α-actinin, actin, myosin binding protein C, desmin and myosin light chain 1) was enhanced by increasing concentrations of H_2O_2 (0-10^5 μM) in the Fenton solution. Carbonylation suspended active force generation following aggressive Fenton treatment (10^5 μM H_2O_2), whereas the application of $3*10^3$ μM H_2O_2 decreased pCa_{50} (from 5.74±0.01 to 5.65±0.01; mean±SEM, $P<0.05$) and increased the passive force (from 1.72±0.21 kN/m^2 to 2.33±0.22 kN/m^2). None of these changes was influenced by sulfhydryl group reduction. Thus, myofilament carbonylation dysregulates the contractile function in human cardiomyocytes, and may therefore mediate the contractile dysfunction during oxidative stress.

Keywords: carbonylation, isolated human cardiomyocytes, contractile function, oxidative stress

1. Introduction

Oxidative stress, a state characterized by excessively produced reactive oxygen and/or nitrogen species (ROS/RNS), plays a central role in the pathogenesis of various cardiovascular diseases, including chronic heart failure [1,2], post-infarction remodeling [3] and diabetic cardiomyopathy [4].

ROS/RNS induce oxidative protein modifications at different subcellular levels, *e.g.* in the myofilaments, resulting in a contractile dysfunction. Protein carbonylation, one of the most commonly used biomarkers of severe oxidative stress [5], is an irreversible oxidative modification that develops when reactive aldehydes or ketones undergo addition to amino acid residues [6]. Primary protein carbonylation occurs in metal-catalyzed oxidation on proline, arginine, lysine and threonine residues [7], whereas a secondary mechanism involves the addition of reactive lipid aldehydes to proteins [8,9]. Metal-catalyzed oxidation results in the production of hydroxyl radicals through the participation of hydrogen peroxide (H_2O_2) and iron(II) cations in the Fenton

reaction [10,11], where hydroxyl radical production can be confirmed by electron paramagnetic/spin resonance spectroscopy [12]. Additionally, this system is widely used for the *in vitro* induction and investigation of protein carbonylation [13].

Protein carbonylation has been implicated as a pathogenic factor in diabetic cardiomyopathy [14], in myocardial ischemia [15], during reperfusion following ischemia [16], after coronary microembolization [17] and in human heart failure [18]. Protein carbonylation can modulate the function of the carbonylated proteins, and can therefore interfere with the activity of signaling molecules such as annexin or peroxiredoxin [8]. However, few data are available as concerns how myofilament protein carbonylation modulates the contractile function of a single cardiomyocyte in the human heart.

In the present study, Fenton chemistry (in which iron(II), H_2O_2 and ascorbic acid participate) was used to induce the *in vitro* protein carbonylation of human myofibrillar proteins. Functional consequences of this oxidative modification were tested in isometric force measurements in isolated permeabilized left ventricular cardiomyocytes. Fenton reaction-derived hydroxyl radicals increased the degree of contractile protein carbonylation in parallel with a decline in Ca^{2+}-dependent active isometric force and its Ca^{2+} sensitivity. Moreover, protein carbonylation also increased the Ca^{2+}-independent passive force, which suggested a potential mechanism for the pathologic regulation of myocardial stiffness.

2. Methods

2.1 Human left ventricular tissue samples

Human donor hearts from four general organ-donor patients (41- and 46-year-old women, and 53- and 57-year-old men) were stored at -80 °C. The cause of death was cerebral aneurysm rupture or hemorrhagic stroke. The donors did not reveal any sign of cardiac abnormalities and had not received any medication apart from short-term dopamine, noradrenaline and mannisol. The experiments on human tissues complied with the Helsinki Declaration of the World Medical Association and were approved by the Hungarian Ministry of Health (No. 323-8/2005-1018EKU).

2.2 Determination of myofilament protein carbonylation by oxyblot assay

The carbonyl group content of the myofilament proteins was determined with an oxyblot protein oxidation detection kit (Millipore, Billerica, MA, USA). Triton-permeabilized (0.5% Triton-X 100; Sigma-Aldrich, St. Louis, MO, USA) cardiomyocytes were treated at room temperature with isolating solution (control) or with isolating solution supplemented with Fenton reagents (containing 50 μM $FeSO_4$, 6 mM ascorbic acid and various concentrations of H_2O_2 in the range 0-10^5 μM; all chemicals from Sigma-Aldrich, St. Louis, MO, USA). After removal of the reagents by washing, the myocardial tissue samples were dissolved in a modified radio-immunoprecipitation assay solution [pH=8.0; 50 mM Tris-HCl, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 6% sodium dodecylsulfate (SDS), 1% protease inhibitor cocktail; all components from Sigma-Aldrich, St. Louis, MO, USA]. Protein concentrations were determined by dot blot, using bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO, USA) as standard. The concentrations of the protein homogenates were adjusted to 3 mg/ml. The subsequent steps were performed according to the manufacturer's recommendations. Briefly, the carbonyl groups were derivatized to 2,4-dinitrophenylhydrazone through reaction with 2,4-dinitrophenylhydrazine. Polyacrylamide gel electrophoresis (PAGE) was used to separate the derivatized protein samples. After Western immunoblotting, the membranes were probed with the antibodies included in the kit, diluted in phosphate-buffered saline containing 0.1% Tween-20 supplemented with 1% BSA. The bands were visualized by the enhanced chemiluminescence method and evaluated by Image J software (free software from the National Institutes of Health, Bethesda, MD, USA). Band intensities were normalized for the amount of actin visualized by Sypro Ruby protein blot staining (Life Technologies, Carlsbad, CA, USA). All assays were performed in triplicates. Data are shown in relative units (carbonylation index; CI), compared to the control.

2.3 Determination of myofilament protein sulfhydryl group oxidation

Sulfhydryl (SH) content was evaluated by the Ellman reaction. Permeabilized cardiomyocytes were treated at room temperature with Fenton reagent solution (containing ascorbic acid, iron and H_2O_2 in the range 0-10^5 μM), or with 2.5 mM 2,2'-dithiodipyridine (DTDP; Sigma-Aldrich, St. Louis, MO, USA) as a positive control, or with 10 mM dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO, USA) as a negative control. SH content was determined by incubation with the SH-sensitive Ellman 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 405 nm, using a NOVOstar microplate reader, and was considered to be proportional to the SH content. The SH content was determined by means of 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 BSA as a standard. The experiments were performed in triplicates.

2.4 Force measurements in permeabilized cardiomyocyte preparations

Frozen human left ventricular myocardial tissue samples were mechanically disrupted in isolating solution (1 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 4 mM ATP, 10 mM imidazole, pH 7.0), and thereafter permeabilization was performed with 0.5% Triton-X 100 detergent. Single permeabilized cardiomyocytes were attached to two stainless steel insect 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 at a sarcomere length (SL) of 2.3 μm using relaxing [10 mM N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 37.11 KCl, 6.41 mM $MgCl_2$, 7 mM EGTA, 6.94 mM ATP, 15 mM creatine phosphate, pH 7.2] and activating (the same components apart from containing CaEGTA instead of EGTA) solutions. The pCa, i.e. $-\log_{10}[Ca^{2+}]$, of the relaxing solution was 9, and that of the activating solution was 4.75. All solutions contained protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 40 μM leupeptin and 10 μM E-64. All chemicals were from Sigma-Aldrich, St. Louis, MO, USA.

Transfer of the myocyte from the Ca^{2+}-free relaxing solution to the activating solution evoked Ca^{2+} contractures. A quick release–restretch maneuver was performed in the activating solution during single Ca^{2+} contractures when the force reached the maximal value. Consequently, the force dropped from peak level to zero, allowing determination of the total force level (F_{total}), and then immediately started to redevelop. The rate constant of force redevelopment ($k_{tr,max}$) at pCa 4.75 was estimated by fitting the force redevelopment after the restretch to a single exponential. The passive force level ($F_{passive}$) was then determined by the shortening of the original preparation length to 80% in relaxing solution. The active isometric force (F_{active}) was calculated by subtracting $F_{passive}$ from F_{total}. F_{active} and $F_{passive}$ were normalized for the cardiomyocyte cross-sectional area, calculated from the width and height of the cardiomyocyte.

The cardiomyocytes (n=6 cardiomyocytes from three human hearts) were subsequently incubated at room temperature in isolating solution supplemented with increasing concentrations of Fenton reagent [50 μM $FeSO_4$, 6 mM ascorbic acid and gradually increasing concentrations of H_2O_2: 0-10^5 μM; all chemicals from Sigma-Aldrich, St. Louis, MO, USA] for 7 minutes for each concentration to assess the concentration-dependent effects of Fenton reaction-induced protein carbonylation on F_{active} and $F_{passive}$. Identical number of activation–relaxation cycles were performed in control cardiomyocytes (n=6 cardiomyocytes from three human hearts) following incubations in isolating solutions without Fenton reagents to assess cardiomyocyte run-down during repeated activations. Ca^{2+}–force relations were assessed both before (control) and after a single 7-minute Fenton reaction exposure (50 μM $FeSO_4$, 6 mM ascorbic acid, $3*10^3$ μM H_2O_2) at a SL of 2.3 μm (n=6 cardiomyocytes from three human hearts) to determine the consequences of protein carbonylation on the Ca^{2+} sensitivity of isometric force production (pCa_{50}). The steepness of the Ca^{2+}–force relation, the Hill coefficient (n_{Hill}), is characteristic of the interfilament cooperativity within the contractile system. Cardiomyocytes were exposed to 10 mM DTT in relaxing solution for 30 minutes at room temperature, subsequently to the single Fenton administration, to eliminate the mechanical effects of possible reversible modifications, *e.g.* SH oxidation.

2.5 Statistical analysis

The numerical data are given in this work as mean values ± SEM. Student t-tests or analysis of variance (ANOVA) followed by the Bonferroni post hoc test were used to compare the experimental groups, with P values <0.05 being considered statistically significant. Statistical analyses were performed with GraphPad Prism 5.02 software (GraphPad Software, Inc., La Jolla, CA, USA).

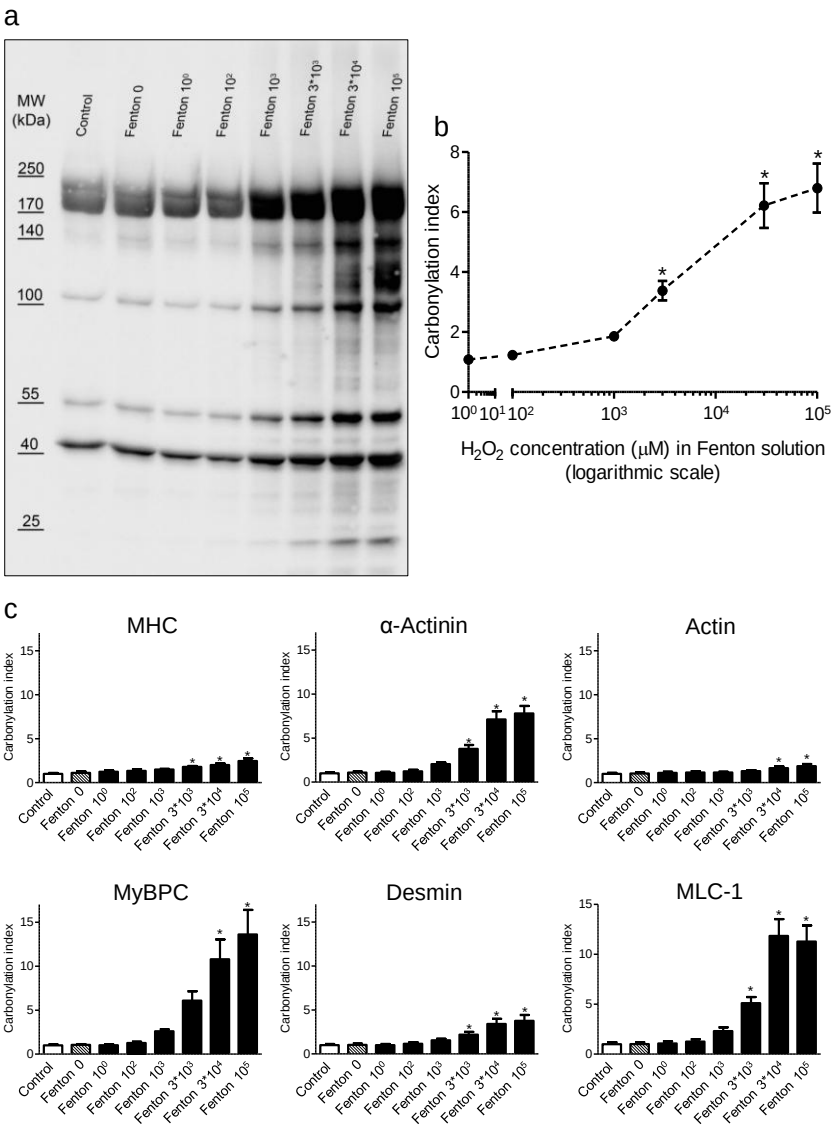

Figure 1. Contractile protein carbonylation induced by the Fenton reaction in permeabilized human cardiomyocytes. Equal amounts of human myocardial proteins were subjected to SDS-PAGE following *in vitro* incubation in the presence of Fenton reagents, and subsequently developed with the protein carbonyl-sensitive oxyblot method. Fenton reaction resulted in the significant carbonylation of myocardial proteins, as evidenced by a representative result revealing increasing signal intensities with increasing concentrations of H_2O_2 in the Fenton mixture (a) and the total carbonylation levels of all myocardial proteins (b). The sensitivity for carbonylation (c) was different for the proteins identified at molecular masses (MW) of: MHC (myosin heavy chain, 200 kDa), MyBPC (myosin binding protein C, 140 kDa); α-actinin (100 kDa), desmin (55 kDa), actin (43 kDa), MLC-1 (myosin light chain-1, 20 kDa). * $P<0.05$ *vs.* Control. Explanation of the nomenclature: as an example Fenton 10^3 indicates Fenton solution containing 10^3 μM H_2O_2.

3. Results

3.1 Myofilament protein carbonylation in human cardiomyocytes

Fenton treatment with increasing H_2O_2 concentrations increased carbonyl group formation in the proteins of the human permeabilized cardiomyocytes, as reflected by the gradual increase seen in the signal intensities in oxyblot assays (Figure 1a). The approximate molecular masses of the affected abundant proteins (i.e. at 20, 43, 55, 100, 140 and 200 kDa) corresponded to those of myofilament proteins: myosin light chain-1 (MLC-1, 20 kDa), actin (43 kDa), desmin (55 kDa), α-actinin (100 kDa), myosin binding protein C (MyBPC, 140 kDa) and myosin heavy chain (MHC, 200 kDa), respectively. The mean levels of carbonylation for all myocardial proteins (expressed in relative units as the CI, the CI for the control being taken as 1.0 and intensities of all of the other bands being compared with that of the control band on the same gel) suggested a sigmoidal H_2O_2 concentration dependence (in the Fenton mixture) with an apparent saturation around 10^5 μM H_2O_2 (Control: 1±0.05; 0 μM H_2O_2: 1.05±0.06; 10^0 μM H_2O_2: 1.08±0.06; 10^2 μM H_2O_2: 1.27±0.07; 10^3 μM H_2O_2: 1.86±0.11; $3*10^3$ μM H_2O_2: 3.38±0.32; $3*10^4$ μM H_2O_2: 6.22±0.75; 10^5 μM H_2O_2: 6.80±0.82; in CI units; Figure 1b). The relative protein carbonylation appeared to be higher than the average for the proteins at the molecular masses of MLC-1, MyBPC, and α-actinin, whereas it was less than the average for the proteins at the molecular masses of desmin, actin, and MHC (Figure 1c).

3.2 High dose Fenton reagent oxidizes the sulfhydryl groups of myocardial proteins

To investigate whether exposure of the cardiomyocytes to Fenton reaction-derived radicals led not only to protein carbonylation, but also to SH

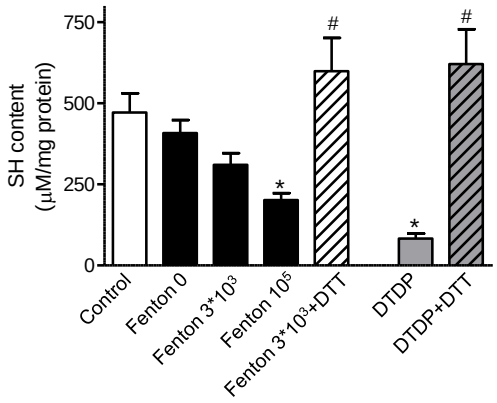

Figure 2. Radicals produced by the Fenton reaction oxidize protein sulfhydryl (SH) groups. The Fenton reaction at 10^5 μM H_2O_2 concentration induced SH oxidation as evidenced by the relative SH group content in Ellman assays. SH oxidation was prevented by the antioxidant dithiothreitol (DTT, 10 mM). 2,2′-dithiodipyridine (DTDP) and DTT after DTDP were used as internal controls. * P<0.05 *vs.* Control; # P<0.05 *vs.* before DTT

group oxidation of the myocardial proteins, the Ellman assay was utilized. *In vitro* exposure to 2.5 mM DTDP served as a positive control. DTT treatment (10 mM) was used to test the reversibility of SH group oxidation. Figure 2 illustrates that Fenton treatment displayed an SH group oxidizing effect (from the control of 471.6±59.1 μM/mg protein to 201.6±21.4 μM/mg protein), which was significant at the highest applied H_2O_2 concentration (Fenton 10^5), but not at the lower concentrations (Fenton 0: 408.1±40.5 μM/mg protein; and at Fenton $3*10^3$: 310.4±36.1 μM/mg protein). DTDP treatment decreased the SH group content even further (to 83.1±15.3 μM/mg protein), whereas the SH group oxidation was reversed by the antioxidant DTT treatment both after Fenton treatment and after DTDP application (599.3±102.1 μM/mg protein and 621.2±107.3 μM/mg protein, respectively). Hence, not only the relative level of protein carbonyls, but also the level of SH groups was a function of the Fenton treatment.

3.3 Protein carbonylation reduces the isometric contractile force in a concentration-dependent manner

To investigate the mechanical consequences of myofilament protein carbonylation, isolated permeabilized human cardiomyocytes (*n*=6) were treated with Fenton solution in a mechanical force measurement set-up similarly as in the biochemical assays. The concentration-dependent effects of the Fenton reaction on isometric active force production were first obtained (Figure 3b). Time control experiments (*n*=6) were also performed to evaluate the run-down of cardiomyocyte contractile performance resulting in a non-significant decrease in relative force to 87.4±2.8% following 10 repeated activations. As reflected by the microscopic

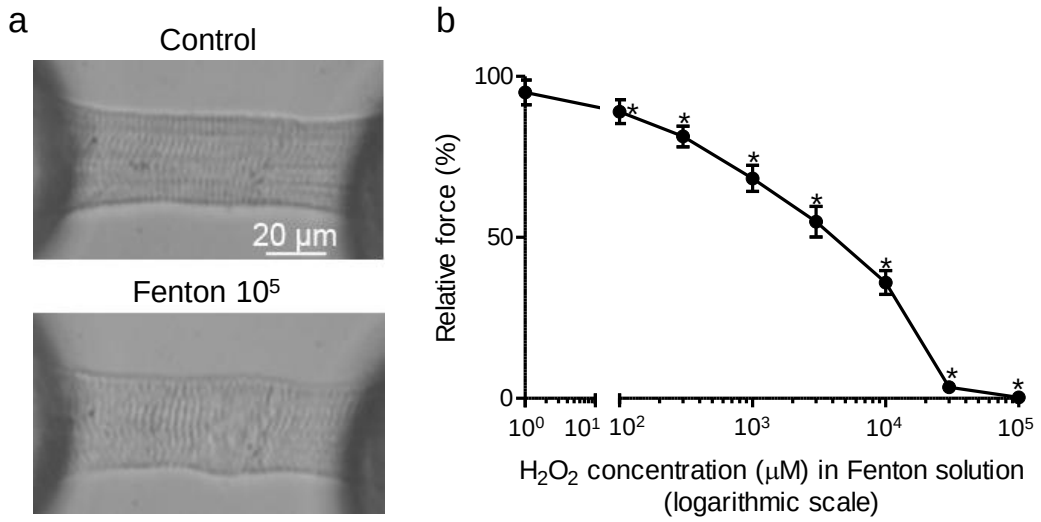

Figure 3. Incubation in Fenton solution did not evoke microscopic alterations, but decreased F_{active}. The Fenton reaction did not induce light-microscopically visible structural changes in the cardiomyocytes mounted on the mechanical force-measuring set-up (a). The carbonylation induced by the Fenton mixtures resulted in a gradual decline in the F_{active} of the Fenton-treated cardiomyocytes (*n*=6) (b). (*n:* number of cardiomyocytes from three human hearts) * P<0.05 *vs.* initial activation

photographs, no major structural changes were seen at any time throughout the experiments (Figure 3a). Nevertheless, a gradual decline in the Ca^{2+}-dependent active force (F_{active}) was observed in parallel with increasing oxidative stress [i.e. with increasing concentrations of H_2O_2, in the presence of constant iron(II) and ascorbic acid concentrations (Control: 100%; Fenton 0: 98.9±3.3%; Fenton 10^0: 95±3.9%; Fenton 10^2: 89.1±3.7%; Fenton 10^3: 68.3±4.1%; Fenton $3*10^3$: 54.9±4.7%; Fenton 10^4: 36±3.7%; Fenton $3*10^4$: 3.5±1.6%; Fenton 10^5: 0.3±0.1%; in relative units, Figure 3b)]. This decrease in F_{active} reflected the increase in the CI for myocardial proteins in this H_2O_2 concentration range (Figure 1b).

3.4 Impaired contractile performance as a result of myofilament carbonylation in human cardiomyocytes

A group of isolated human cardiomyocytes (n=6) was subjected to a single exposure to Fenton solution containing $3*10^3$ μM H_2O_2 to test its effects on the Ca^{2+} sensitivity of force production (pCa_{50}), F_{active} and $F_{passive}$. Subsequent DTT exposures were used to test the reversibility, and to estimate the SH-sensitive components of the observed mechanical changes. A clear reduction in the Ca^{2+} sensitivity of force production was observed following incubation in the presence of this Fenton solution, which was reflected by the rightward shift of the Ca^{2+}–force relation and a significant decrease in pCa_{50} [pCa_{50}(Control): 5.74±0.01; pCa_{50}(Fenton): 5.65±0.01; P<0.05]. Besides the decreased Ca^{2+} sensitivity of force production (Figure 4a,b), a reduced F_{active} (Figure 4c) and an increased $F_{passive}$ (Figure 4d) were detected [F_{active}(Control): 11.39±1.63 kN/m²; F_{active}(Fenton): 8.66±1.06 kN/m²; $F_{passive}$(Control): 1.72±0.21 kN/m²; $F_{passive}$(Fenton): 2.33±0.22 kN/m²]. The rate constant of tension redevelopment ($k_{tr,max}$) and the Hill coefficient (n_{Hill}) were not affected by the *in vitro* induced carbonylation [$k_{tr,max}$(Control): 0.68±0.06 1/sec; $k_{tr,max}$(Fenton): 0.57±0.02 1/sec; n_{Hill}(Control): 2.42±0.05; n_{Hill}(Fenton): 2.31±0.06]. Subsequent DTT treatment affected none of these parameters [pCa_{50}(DTT): 5.63±0.01; F_{active}(DTT): 7.14±1.1 kN/m²; $F_{passive}$(DTT): 2.44±0.28 kN/m²; $k_{tr,max}$(DTT): 0.55±0.07 1/sec; n_{Hill}(DTT): 2.43±0.1; P>0.05 *vs.* Fenton; P<0.05 *vs.* Control].

4. Discussion

As far as we are aware, this is the first reported study of the direct effects of protein carbonylation on the contractile function of human

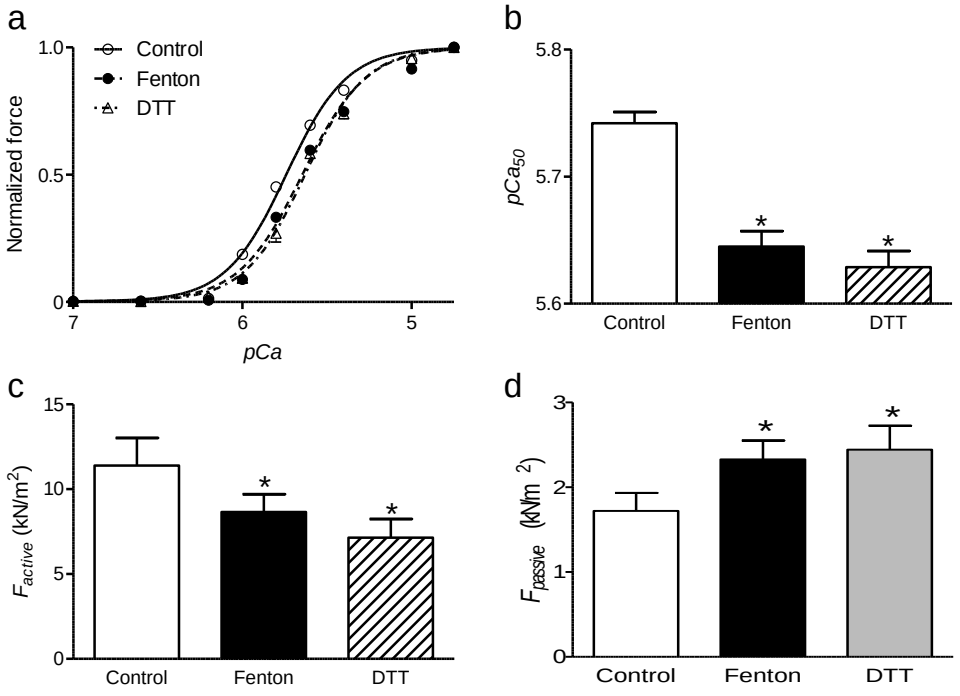

Figure 4. The effects of carbonylation on the mechanical properties of human cardiomyocytes. The Ca^{2+}–force relations (a) and the mean pCa_{50} values (b) illustrate that exposure to the Fenton solution containing $3*10^3$ μM H_2O_2 resulted in a decreased Ca^{2+} sensitivity of force production. Carbonylation induced a decrease in F_{active} (c) and an increase in $F_{passive}$ (d). DTT treatment had no effects on either mechanical parameter. Error bars are indicated where larger than symbol size. n=6 cardiomyocytes from three human hearts * P<0.05 *vs.* Control

cardiomyocytes. To this end, Fenton chemistry was used to produce hydroxyl radicals and to induce protein carbonylation in permeabilized left ventricular cardiomyocytes *in vitro*, an approach which allowed the characterization of the relationship between the level of myofilament carbonylation and quantitative indices of isometric force production. Interestingly, the carbonylation-evoked mechanical dysfunction was not accompanied by visible microscopic alterations in the cardiomyocyte structure. Hence, our results suggest that myofilament protein carbonylation disturbs the fine regulation of cardiomyocyte contractility.

Several different types of post-translational oxidative contractile protein modifications (*e.g.* SH group oxidation, protein nitration, protein carbonylation, *etc.*) have been reported as possible mediators of a contractile dysfunction during oxidative stress in the heart [19]. The mechanical consequences have also been elucidated for some, but not all of the above protein changes. Superoxide anion exposure, for instance reduced F_{active} in a concentration-dependent manner, but had no effect on the Ca^{2+} sensitivity of force production in the rat right ventricular trabeculae [20]. Similarly to superoxide anion, peroxynitrite-induced protein nitration impaired the contractile function in isolated human and rat cardiomyocytes [21,22]. A high degree of myofilament SH oxidation was earlier demonstrated to decrease not only myofilament force production, but also its Ca^{2+} sensitivity in permeabilized human cardiomyocytes [23]. However, practically no information is currently available as concerns the mechanical effects of contractile protein carbonylation in human cardiomyocytes.

In a rat model of right heart failure, serotonin-mediated right ventricular protein carbonylation was considered to be an important pathophysiologic factor [24]. Moreover, myofibrillar actin carbonylation was recognized both after experimental ischemia–reperfusion and following H_2O_2 exposure in isolated rat hearts [16]. The same investigators also reported on the carbonylation of the thin myofilament protein, tropomyosin in porcine and canine hearts in response to coronary microembolizations, the degree of tropomyosin oxidation correlating with the contractile dysfunction observed [17]. Further, the carbonylation of MyBPC, MHC and actin has been implicated in post-ischemic murine models [25,26].

Bagatini *et al.* reported an increase in the serum protein carbonylation of human patients with acute myocardial infarction and in others with risk factors for acute myocardial infarction [27]. Elevated plasma protein carbonyl levels were likewise detected during coronary artery bypass grafting, both after the initiation of ischemia and during the reperfusion period [28]. The carbonylation of tropomyosin has been correlated with the contractile impairment in human heart failure, and this type of protein modification has also been regarded as a marker of severe oxidative stress [18]. Thus, myofilament protein carbonylation appears to be central in the pathogenesis of the myocardial dysfunction during oxidative stress.

In the present study, the effects of contractile protein carbonylation were provoked *in vitro* by a hydroxyl radical-generating Fenton reaction in human permeabilized cardiomyocytes. This reaction is observed in failing cardiac myocytes [29] and during ischemia–reperfusion injury [30], and the Fenton reaction is widely employed to produce hydroxyl radicals by the reaction of iron(II) and H_2O_2 for *in vitro* experiments [13,31,32]. The advantage of the combination of this technique with the study of isolated, permeabilized cardiomyocytes is that it excludes interference with membrane components, intracellular organelles and elements of the Ca^{2+} homeostasis [33].

Oxidative protein modifications other than protein carbonylation cannot be excluded for hydroxyl radical exposures [13,34]. In our studies, we paid specific attention to SH oxidation, and observed that a significant level of SH oxidation could be detected only when an extremely high concentration of H_2O_2 was applied in the Fenton system. Moreover, despite the SH oxidation, DTT did not prevent the alterations in the cardiomyocyte mechanics, suggesting that at the lower H_2O_2 concentrations the underlying molecular mechanism is not related to SH oxidation. On the other hand, the contractile proteins of the human cardiomyocytes (similarly to those in animal hearts) proved to be highly sensitive to carbonylation. Exposure to the increasing concentrations of H_2O_2 in the Fenton reagent enhanced the level of protein carbonylation and reduced the maximal isometric force in a graded fashion. It is unlikely that the highest concentrations of radicals produced by this method are present under physiological conditions [35]. Nonetheless, free radical concentrations might reach levels sufficiently high to provoke relevant levels of protein carbonylation *in vivo* in diffusion-limited compartments. Hydroxyl radicals are highly reactive, acting immediately on the first cell components they meet [35]. In our *in vitro* study, hydroxyl radicals were generated in the direct vicinity of cardiomyocytes and modified a number of myofilament proteins. Collectively, it is uncertain whether the carbonylation of a selected single protein alone or that of a group of proteins in

combination was responsible for the observed mechanical changes.

Oxidative protein modifications resulting in a gain or loss of function or targeted degradation may lead to impaired cellular functions or the accumulation of modified proteins [36]. These changes can dysregulate the contractile protein machinery of cardiomyocytes during conditions of oxidative stress. In our present experiments, protein carbonylation decreased active force production and its Ca^{2+} sensitivity similarly as observed earlier in the residual cardiomyocytes of the infarcted zone in post-infarction murine hearts [26]. These changes may contribute to the development of weaker myocardial contractions and hence to a systolic dysfunction. We also report here that myofilament protein carbonylation increases the Ca^{2+}-independent passive force, thereby implicating a potential molecular mechanism for increased cardiomyocyte stiffness in a diastolic dysfunction for the human heart [37].

Importantly, a reduced Ca^{2+} responsiveness and/or contractile dysfunction are frequently referred to in clinical conditions in association with oxidative stress. In a post-infarction murine model, left ventricular remodeling and dysfunction were prevented by the hydroxyl radical scavenger dimethylthiourea [38]. Moreover, myocardial stunning is also characterized by excessive oxidative stress and reduced myofilament Ca^{2+} sensitivity [39,40]. The present results suggest that myofilament carbonylation may be regarded as a potential link between myofilament oxidation and the contractile dysfunction in the above conditions.

5. Acknowledgments

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