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The peroxynitrite evoked contractile depression can be partially reversed by antioxidants in human cardiomyocytes

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Abstract

In this study, we aimed to determine the contribution of peroxynitrite-dependent sulfhydryl group (SH) oxidation to the contractile dysfunction in permeabilized left ventricular human cardiomyocytes using a comparative approach with the SH-oxidant 2,2′-dithiodipyridine (DTDP). Additionally, different antioxidants: dithiothreitol (DTT), reduced glutathione (GSH) or N-acetyl-L-cysteine (NAC) were employed to test reversibility. Maximal isometric active force production (F_o) and the maximal turnover rate of the cross-bridge cycle ($k_{tr,max}$) illustrated cardiomyocyte mechanics. SH oxidation was monitored by a semi-quantitative Ellman's assay and by SH-specific protein biotinylation. Both peroxynitrite and DTDP diminished F_o in a concentration-dependent manner ($EC_{50,peroxynitrite}$ = 49 μM; $EC_{50,DTDP}$ = 2.75 mM). However, $k_{tr,max}$ was decreased only by 2.5-mM DTDP, but not by 50 μM peroxynitrite. The diminution of F_o to zero by DTDP was paralleled by the complete elimination of the free SH groups, while the peroxynitrite-induced maximal reduction in free SH groups was only to 58 ± 6% of the control (100%). The diminutions in F_o and free SH groups evoked by 2.5-mM DTDP were completely reverted by DTT. In contrast, DTT induced only a partial restoration in F_o (ΔF_o:~13%; $P < 0.05$) despite full reversion in protein SH content after 50 μM peroxynitrite. Although, NAC or DTT were equally effective on F_o after peroxynitrite exposures, NAC or GSH did not restore F_o or $k_{tr,max}$ after DTDP treatments. Our results revealed that the peroxynitrite-evoked cardiomyocyte dysfunction has a small, but significant component resulting from reversible SH oxidation, and thereby illustrated the potential benefit of antioxidants during cardiac pathologies with excess peroxynitrite production.

Keywords: peroxynitrite • sulfhydryl groups • nitrosative/oxidative stress • ischaemic reperfusion injury • cardiomyocyte • heart

Introduction

Oxidative and nitrosative radicals have been associated with the development of myocardial tissue injury during chronic heart failure, reperfusion that follows ischaemia and in response to inflammatory cytokines or cardiotoxic drugs [1–9]. Under these pathologic conditions, various protein and lipid molecules serve as targets for the accumulating oxidative and/or nitrosative agents [10, 11]. Proteins of the sarcomere are of special interest because their alterations will influence the structure and/or the regulation of the interaction between the thin and the thick myofilaments, and thereby will affect directly the conversion of chemical energy into force and shortening [12, 13].

Peroxynitrite, a metabolite of nitric oxide, is frequently cited as one of the most damaging nitrosative agents [2, 4, 14], although peroxynitrite is not necessarily toxic as basal peroxynitrite formation may have several physiological roles [15]. Oxidation of protein tyrosine residues by peroxynitrite results in nitrotyrosine formation, which is considered as a hallmark of the peroxynitrite-evoked protein damage [16]. However, reactive nitrogen species also react with the thiol groups (SH) of cysteinil residues and thus, may induce the formation of disulfide bonds [8, 17, 18]. It is worthy for consideration, that while the generation of nitrotyrosine side chains is considered to be irreversible, the oxidation of SH groups is

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reversible. Hence, antioxidants may theoretically prevent or revert, at least part of the peroxynitrite-evoked protein changes [19].

Importantly, both the tyrosine residues [2] and the protein SH groups were found to undergo marked changes during ischaemia and reperfusion [20, 21]. Moreover, both the amounts of nitration and SH oxidation of proteins have been associated with the contractile depression in various myocardial preparations of animal and human hearts [4, 5, 22, 23]. Nevertheless, the relative contribution of SH oxidation to the peroxynitrite-evoked contractile dysfunction is unknown.

In this study, we made attempts to elucidate the SH-oxidizing potential of peroxynitrite, and to determine the reversibility of peroxynitrite-dependent mechanical alterations in human cardiomyocytes. To these ends, permeabilized left ventricular cardiomyocytes were incubated in the presence of peroxynitrite, which has hypothetically effects both on protein SH groups and on tyrosine residues. In parallel assays, 2,2′ dithiodipyridine (DTDP) was employed as a selective SH-oxidant [24]. In addition, dithiothreitol (DTT), reduced glutathione (GSH) or N-acetyl-cysteine (NAC) were applied as reducing agents to test reversibility. Maximal Ca^{2+}-activated force (F_o) and the cross-bridge-specific rate constant of force redevelopment at saturating Ca^{2+} concentration ($k_{tr,max}$) were monitored along with these incubations, and were considered as indicators of the actin-myosin interactions. Additionally, the Ca^{2+}-independent passive force component ($F_{passive}$) was also assessed. Furthermore, biochemical assays were performed to determine the SH-specificity of the reagents and to evaluate the involvement of myocardial proteins in parallel with the mechanical changes.

Our results illustrate myofilament SH-oxidation as a potential mechanism contributing to the peroxynitrite-evoked mechanical dysfunction of human cardiomyocytes. Moreover, our data are also suggestive for a complex interplay between the chemical characteristics of the applied oxidative and reducing agents and the mechanical function of human cardiomyocytes.

Materials and methods

Ethical approval

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) and by the Institutional Ethical Committee at the University of Debrecen, Hungary (No. DEOEC RKEB/IKEB 2553-2006).

Human left ventricular tissue samples, permeabilized cardiomyocytes

Human donor hearts obtained from 5 general organ donor patients (male and female donors between 37 and 56) were explanted to obtain pulmonary and aortic valves as homografts for cardiac surgery. The donors did not reveal any sign of cardiac abnormalities and had not received any medication except short-term dobutamine and furosemide. The cause of death was cerebral contusion and cerebral haemorrhage due to accidents or subarachnoid haemorrhage. Biopsies were transported in cardioplegic solution (pH 7.4; in mM): NaCl 110, KCl 16, $MgCl_2$ 1.6, $CaCl_2$ 1.2, $NaHCO_3$ 5 and kept at 4°C for ~1–4 hrs before being frozen in liquid nitrogen and stored at −80°C.

Frozen tissue samples were defrosted and mechanically disrupted in isolation solution (composition in mM: $MgCl_2$ 1, KCl 145, EGTA 2, ATP 4, imidazole 10; pH 7.0). The resultant suspension was incubated in isolation solution supplemented with 0.5% Triton X- 100 (Sigma, St. Louis, MO, USA) for 5 min. to permeabilize all the membranous structures. The preparations were washed three times (centrifugation with 1000 *rpm* for 1 min. and subsequently kept at 4°C till the following experiments.

Mechanical properties of cardiomyocytes, *in vitro* applications of oxidative and reducing agents

Permeabilized single cardiomyocyte preparations were mounted between two thin needles with silicone adhesive (Dow Corning, Midland, MI, USA) while viewed under an inverted microscope (Axiovert 135, Zeiss, Germany) [25, 26]. The advantage of these preparations is that they present negligible diffusion obstacles, allowing almost instantaneous equilibration of oxidative and reducing agents between the bathing medium and the proteins of the cardiomyocytes. One needle was attached to a force transducer (SensoNor, Horten, Norway) and the other to an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada). The force measurements were performed at 15°C, and the average sarcomere length was adjusted to 2.2 μm as described previously [27].

The compositions of the relaxing and activating solutions used during force measurements were calculated as described earlier [28, 29]. The pCa ($-\log[Ca^{2+}]$) values of the relaxing and activating solutions (pH 7.2) were 9 and 4.75, respectively. All the solutions for force measurements contained (in mM): Mg^{2+} 1, MgATP 5, phosphocreatine 15 and N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid 100. The ionic equivalent was adjusted to 150 mM with KCl resulting in an ionic strength of 186.

F_o, $F_{passive}$ and $k_{tr,max}$ were determined as described earlier [25]. These contractile parameters were measured under both control (before peroxynitrite or DTDP) and test conditions (*i.e.* after exposure to various oxidative and/or reducing agents). Concentrated stock solutions of peroxynitrite (Calbiochem, San Diego, CA, USA) were prepared based on peroxynitrite concentration determination by absorbance measurements at 302 nm. The pH in stock solutions was adjusted to 11 (by KOH) to oppose peroxynitrite decomposition. A single volume of 20 μl from these stock solutions was rapidly introduced into a droplet (180 μl) of relaxing solution (pH 7.2, T = 22°C), which surrounded each myocyte preparation in the mechanical set-up. This approach resulted in nominal peroxynitrite concentrations ranging from 1 to 1000 μM, which decreased quickly because of spontaneous degradation (half-life: less than 3 sec. in our system). Peroxynitrite exposure was terminated following 60 sec. of incubation. Control force measurements suggested that non-specific effects due to hydrogen peroxide contamination or the by-products of peroxynitrite (*i.e.* nitrite or nitrate) have a very limited role, if any in our system. Incubations in the presence of other oxidative and reducing agents were also performed in droplets of 200 μl volumes of relaxing solutions. Incubations with DTDP (Sigma) lasted for 2 min., however with 10-mM DTT (Eastman Kodak Company, Rochester, MN, USA), 10-mM GSH or 100-mM GSH (Sigma) or 10-mM NAC or 100-mM of NAC (Sigma) these were extended for 30 min. (all at 22°C). Results of control measurements and literature data suggested that these durations were sufficient to reach steady-state changes in the SH status of our preparations (data not shown).

To assess the concentration dependences of peroxynitrite or DTDP on *Fo* the cardiomyocytes were exposed to a series of solutions with various concentrations of peroxynitrite or DTDP at *p*Ca 9.0, and subsequently to *p*Ca 4.75 without peroxynitrite or DTDP. In a different set of experiments, the determination of control F_o was followed by the exposures to 2.5-mM DTDP or 50-μM peroxynitrite and thereafter DTT, GSH or NAC was applied to test reversibility. To determine the effect of run-down and the stability of our preparations control measurements (in $n = 4$–6 cardiomyocytes) with the same number of activations as with the oxidative and/or reducing agents were performed without any chemicals. During these control experiments F_o decreased at maximum to 82 ± 1% (mean ± S.E.M.; $P < 0.05$), $k_{tr,max}$ to 81 ± 5% ($P < 0.05$), while $F_{passive}$ was stable (*i.e.* at the end of test runs it was 100 ± 3% of the initial value, $P > 0.05$).

Quantitative determination of the SH status of myofilament proteins

To determine the SH content, permeabilized cardiomyocytes (prepared similarly to the mechanical measurements) were treated with the different oxidative and/or reducing agents in isolation buffer at a protein concentration of 5 mg/ml at 22°C. Then SH content was determined by incubation with the SH-sensitive Ellman's reagent (5,5′-dithio-bis(2-nitrobenzoic acid); Sigma)) for 15 min. at 22°C [30]. The absorbance of the solutions at 412 nm was considered to be proportional to their SH contents. The samples were assessed *via* calibration curves (standards: N-acetyl-L-cysteine and reduced glutathione; both from Sigma) fitted to a single exponential, and the SH contents of the cardiac samples were calculated.

Qualitative analysis of SH groups oxidized in myofilament proteins

To analyse the sensitivity of certain myocardial proteins to SH oxidizing agents, permeabilized cardiomyocytes were incubated in the presence of DTDP (up to 2.5 mM) or peroxynitrite (up to 1000 μM) in relaxing solution and subsequently in the presence of DTT, GSH or NAC to test reversibility. Then, the reagents were removed by three washing steps and the protein concentrations were adjusted to 5 mg/ml. Subsequently, preparations were incubated in the presence of 60 μM (+)-biotinyliodoacetamidyl-3, 6-dioxaoctanediamine (Pierce, Rockford, IL, USA) at 22°C for 90 min. to biotinylate the SH groups of the proteins. After biotinylation, the preparations were washed in isolation buffer three times, and boiled in SDS-PAGE loading buffer (Sigma). The protein concentrations were tested by a dot blot-based method; thereafter 25 μg protein homogenates were applied to 10% gels or to 6–18% gradient gels (Biorad, Hercules, CA, USA) and subsequently transferred to nitrocellulose membranes. The membranes were blocked in 5% milk powder (1 hr) and then incubated with a streptavidin-peroxidase conjugate (Vector Laboratories, Burlingame, CA, USA) for 30 min. Bands representing biotinylated proteins at their free (reduced) SH groups were recorded on autoradiographic films (Primax RTG-B, Berlin, Germany), resulting in dark signals. Signal intensities were considered to be proportional with the free SH group contents of the respective proteins.

Data analysis

Statistical significance was calculated by analysis of variance (ANOVA, repeated measures) and, where applicable, by Student's t-test. Values are given as means ± S.E.M. The number of experiments in each group varied between 3 and 31 from three to five different hearts. Statistical significance was accepted at $P < 0.05$.

Results

Both peroxynitrite and DTDP decreased F_o to zero in permeabilized human ventricular cardiomyocytes in a concentration-dependent manner (Fig. 1A). In case of peroxynitrite, the force diminution was noticed in the μM concentration range ($EC_{50,peroxynitrite} = 49$ μM), while DTDP exerted its mechanical effect at higher concentrations ($EC_{50,DTDP} = 2.75$ mM). To elucidate the SH-oxidizing effect of peroxynitrite and DTDP, parallel biochemical assays were performed with the SH-sensitive Ellman's reagent. Of note, the experimental conditions, including temperature for the Ellman's assays and mechanical measurements, were comparable. Figure 1B illustrates that increasing concentrations of DTDP decreased the SH content of the myocardial proteins to zero along with the diminution of force. On the contrary, peroxynitrite-evoked significant SH oxidation only at very high concentrations: the fraction of reduced SH groups decreased to 58 ± 7% ($P < 0.05$) of that of the untreated control (100%) following the application of 1-mM peroxynitrite.

Previous experimental studies revealed interactions between protein kinase A-mediated intracellular signalling and protein SH oxidation [31, 32]. To address if the short-term dobutamie premedication had an effect on SH oxidation of the myocardial proteins, we compared the SH contents of the myocardial proteins before and after incubations in the presence of 10-mM DTT, 100-mM NAC or 100-mM GSH. Results of these assays did not differ significantly from those of untreated controls and from each other, and collectively they gave a relative level of SH content of 101.7 ± 8.2%. Hence, these data argued against a hypothetical protein oxidation by dobutamine.

Having established that SH oxidation associates with the deterioration of contractile force, the parameters of cardiomyocyte mechanics (F_o, $k_{tr,max}$ and $F_{passive}$) were investigated at a single peroxynitrite concentration (50 μM) resulting in about half maximal reduction in F_o (Fig. 2). F_o declined to 56 ± 4% of the control after the application of 50 μM peroxynitrite (Fig. 2B), however $F_{passive}$ and $k_{tr,max}$ did not change significantly (Fig. 2C and D). To estimate the relative contribution of SH oxidation besides other types of potential protein modifications to the reduction in F_o, SH-specific reducing agents were employed to reverse SH oxidation. Figure 2B illustrates that indeed both 10-mM DTT and 10-mM NAC were able to evoke partial, although significant, increases in F_o in peroxynitrite-treated cardiomyocytes, suggesting that this increase was specific for the reduction of protein SH groups. F_o was increased to 69 ± 4% of the control by 10-mM DTT ($P < 0.05$ *versus* $F_{o,peroxynitrite}$), and the relative increase in F_o was to 71 ± 7% of the control by 10-mM NAC ($P < 0.05$ *versus* $F_{o,peroxynitrite}$). $F_{passive}$ did not change following 10-mM DTT or

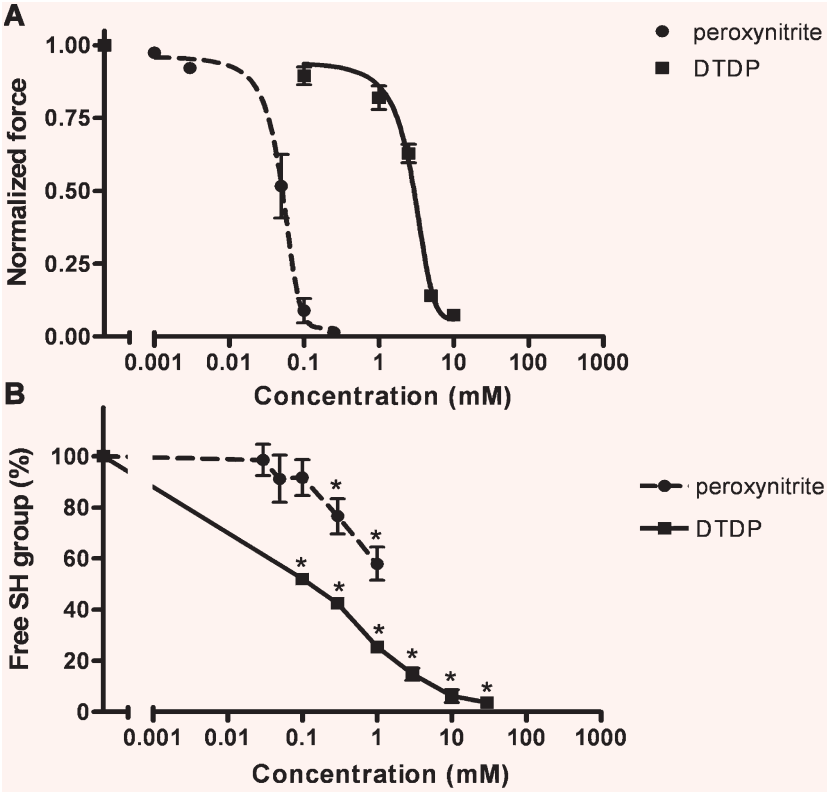

Fig. 1 Changes in F_0 and human myocardial protein SH content with the application of increasing concentrations of peroxynitrite or DTDP. (**A**) The concentration-effect relation of peroxynitrite (•) and DTDP (▪) on F_0. Peroxynitrite and DTDP, both abolished F_0 in a concentration-dependent manner but in different concentration ranges. (**B**) The concentration-effect relation of peroxynitrite (•) and DTDP (▪) on the relative amount of myocardial-free SH groups (untreated control: 100%). DTDP oxidized all myocardial protein SH groups in the same concentration range where F_0 diminished. In contrast, peroxynitrite evoked only a partial reduction in the amount of free SH groups even at the highest employed peroxynitrite concentration. Symbols denote means ± S.E.M. when larger than symbol size. The curves connecting the mean values in A and B were constructed by eye and serve illustrative purposes (n = 20 cardiomyocytes for peroxynitrite; n = 4 cardiomyocytes for DTDP). * Marks significant differences *versus* controls.

10-mM NAC in peroxynitrite-treated cardiomyocytes (Fig. 2C). Of note, $k_{tr,max}$ decreased slightly following 10-mM DTT or 10-mM NAC applications in peroxynitrite-treated cardiomyocytes. However, based on control experiments with repeated activations in the absence of any chemicals, this small decrease in $k_{tr,max}$ was probably related to preparation run-down.

The partial contribution of SH oxidation to the peroxynitrite-evoked force reduction was confirmed by biochemical methods (Fig. 3). Results of Ellman's assays showed that 1-mM peroxynitrite (a peroxynitrite concentration with maximal SH oxidative effect, Fig. 1B) decreased the free SH content to 58 ± 7%, which was completely reversed by 10-mM DTT. SH content after 1-mM peroxynitrite + 10-mM DTT was 94 ± 5% ($P < 0.05$ *versus* 1-mM peroxynitrite). Surprisingly, when applied at the 10-mM concentration the effects of GSH or NAC did not reach significance on the overall free SH content of the myocardial protein preparations. SH content after 1-mM peroxynitrite + 10-mM GSH was 65 ± 6%, and SH content after 1-mM peroxynitrite + 10-mM NAC was 64 ± 6% ($P > 0.05$ *versus* 1-mM peroxynitrite). However, when the same reducing agents were employed at a concentration of 100 mM, both of them reduced protein SH groups effectively. Protein SH content after 1-mM peroxynitrite + 100-mM GSH was 105 ± 15% and SH content after 1-mM peroxynitrite + 100-mM NAC was 118 ± 14% ($P < 0.05$ *versus* 10-mM peroxynitrite; Fig. 3A). An effort was also made to identify whether certain proteins undergo selective oxidation-reduction cycles during the incubations with the various drug combinations. SH-specific biotinylation, however, suggested a uniform decrease in the free SH group-specific staining intensity of myocardial proteins with different molecular weights at the highest peroxynitrite concentration (1 mM). Moreover, these changes were fully reversed by 10-mM DTT, and only to a smaller degree by 10-mM GSH or 10-mM NAC (Fig. 3B). Therefore, the results of these assays were reminiscent of the results of the Ellmans's test, but failed to identify peroxynitrite-specific selective myocardial protein oxidation.

As a next step, the effects of DTDP-evoked SH oxidation were compared to the peroxynitrite-induced alterations. The Ellman's assay indicated that 2.5-mM DTDP (a DTDP concentration with a comparable effect on F_0 to that of 50 μM peroxynitrite, Fig. 1A) resulted in a robust decrease in the myocardial free SH content (*i.e.* it decreased to 14 ± 2%; Fig. 4A), which was reversed either completely (by DTT) or partially (by GSH or NAC) when the antioxidants were all used at the same 10-mM concentration. SH content after 2.5-mM DTDP + 10-mM DTT was 97 ± 14%, SH content was 34 ± 8% after 2.5-mM DTDP + 10-mM GSH, and it was 30 ± 4% after 2.5-mM DTDP + 10-mM NAC (for all $P < 0.05$ *versus* 2.5-mM DTDP). Moreover, 100-mM GSH or 100-mM NAC reduced 2.5-mM DTDP-oxidized myocardial proteins more effectively than 10-mM GSH or 10-mM NAC. SH content after 2.5-mM DTDP + 100-mM GSH was 79 ± 3%, whereas it was 68 ± 15% after 2.5-mM DTDP + 100-mM NAC (for both $P < 0.05$ *versus* 2.5-mM DTDP). To compare the effects of antioxidants at a similar level of DTDP-evoked SH oxidation as occurred after the application of 1-mM peroxynitrite, the above tests were also repeated

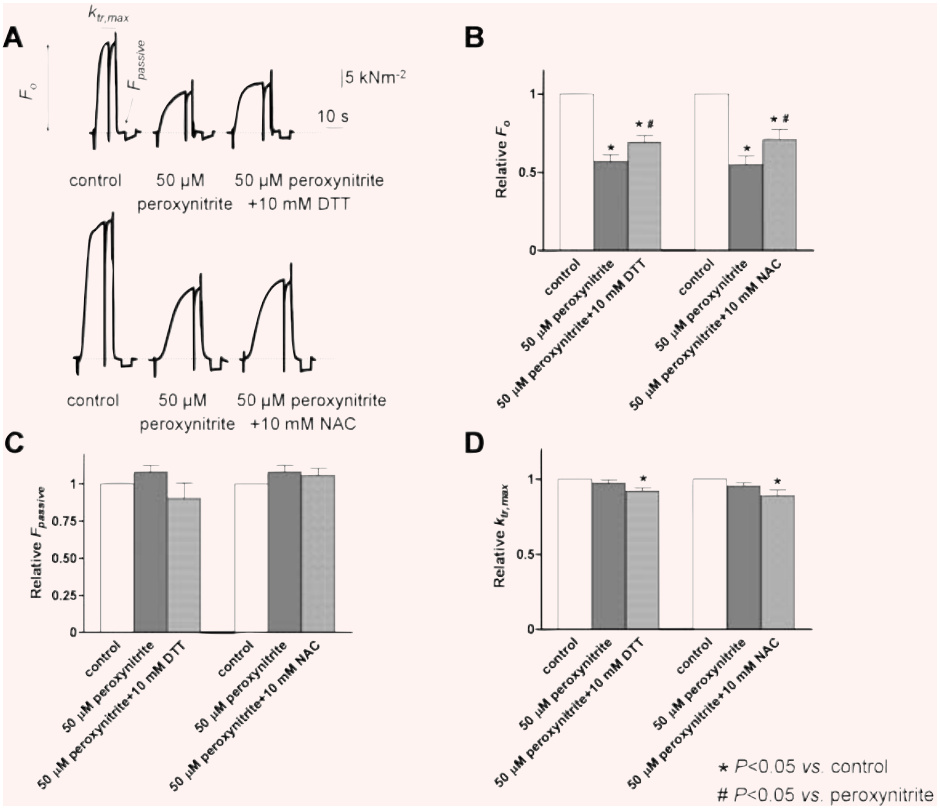

Fig. 2 Partial reversion of the mechanical effects evoked by 50 μM peroxynitrite following the applications of SH-reducing agents. (**A**) Original recordings illustrate the mechanical effects of the sequentially applied 50 μM peroxynitrite and 10-mM DTT (upper panel) and 50 μM peroxynitrite and 10-mM NAC (lower panel). Bar graphs (means ± S.E.M.) illustrate the magnitudes of relative changes in F_o (**B**), $F_{passive}$ (**C**) and in $k_{tr,max}$ (**D**). Peroxynitrite decreased F_o, but did not affect $F_{passive}$ *and* $k_{tr,max}$. DTT or NAC induced only a partial reversion in F_o peroxynitrite. Control values for F_o, $F_{passive}$ and $k_{tr,max}$ were 28.6 ± 1.8 kN/m^2; 2.26 ± 0.18 kN/m^2; 1.02 ± 0.02 1/sec., respectively. The number of cardiomyocytes ranged between 12 and 31 for the different tests.

following incubations in the presence of 0.1-mM DTDP (Fig. 4B). 0.1-mM DTDP decreased myocardial-free SH content to 57 ± 12%, which was reversed completely by 10-mM DTT (*i.e.* to 118 ± 20%). The effects of the other two reducing agents GSH and NAC did not reach significance when they were applied at the 10-mM concentration. SH content was 72 ± 6% after 0.1-mM DTDP + 10-mM GSH, and it was 61 ± 5% after 0.1-mM DTDP + 10-mM NAC (for all $P > 0.05$ *versus* 0.1-mM DTDP). However, when GSH or NAC were employed at a concentration of 100 mM, both of them reduced SH groups completely. SH content was 123 ± 15% after 0.1-mM DTDP + 100-mM GSH, and it was 102 ± 8% after 0.1-mM DTDP + 100-mM NAC (for both $P < 0.05$ *versus* 0.1-mM DTDP). Collectively, the results of Ellman's assays at similar levels of (peroxynitrite-evoked or DTDP-evoked) protein SH oxidation suggested identical characteristics for the employed antioxidants irrespectively of the molecular nature of the oxidizing agents. Interestingly, the assays involving protein biotinylation revealed that DTDP oxidation did not affect all of the myofibrillar proteins uniformly (Fig. 4C). Reduced SH groups in some proteins were apparently more resistant to DTDP (bands still present at 2.5-mM DTDP) than in others. In agreement with the results of Figure 4A, 10-mM DTT apparently reduced the SH groups of all proteins, however 10-mM GSH and 10-mM NAC were only partially effective.

Finally, the effects of 2.5-mM DTDP-evoked SH oxidation on the contractile parameters and on their reversibility were tested (Fig. 5). 2.5-mM DTDP decreased F_o to 64 ± 2% ($P < 0.05$ *versus* control), and $k_{tr,max}$ from a control value of 1.05 ± 0.05 s^{-1} to 0.78 ± 0.05 s^{-1} ($P < 0.05$), and induced a modest increase in $F_{passive}$ in some but not in all experiments. Similarly to the peroxynitrite-evoked SH oxidation, the DTDP-evoked biochemical and mechanical effects were largely reversed by 10-mM DTT, suggesting that DTT treatment may be a preferred choice to assess the contribution of protein SH oxidation to the contractile dysfunction under various experimental conditions. As a matter of the different effectiveness for the employed reducing agents, other proofs were also found. While 10-mM DTT seemed to be suitable to reverse SH oxidation and contractile mechanics, 10-mM GSH or 10mM NAC worsened the mechanical effects of 2.5-mM DTDP: the elevations in $F_{passive}$ were further elevated, the decreased F_o was further decreased, and the $k_{tr,max}$ was slower after GSH and NAC treatments than before.

Discussion

In this study, we investigated the contribution of SH oxidation to the development of peroxynitrite-mediated contractile dysfunction using human permeabilized cardiomyocytes. Results of *in vitro* experiments revealed that the potential of peroxynitrite to evoke

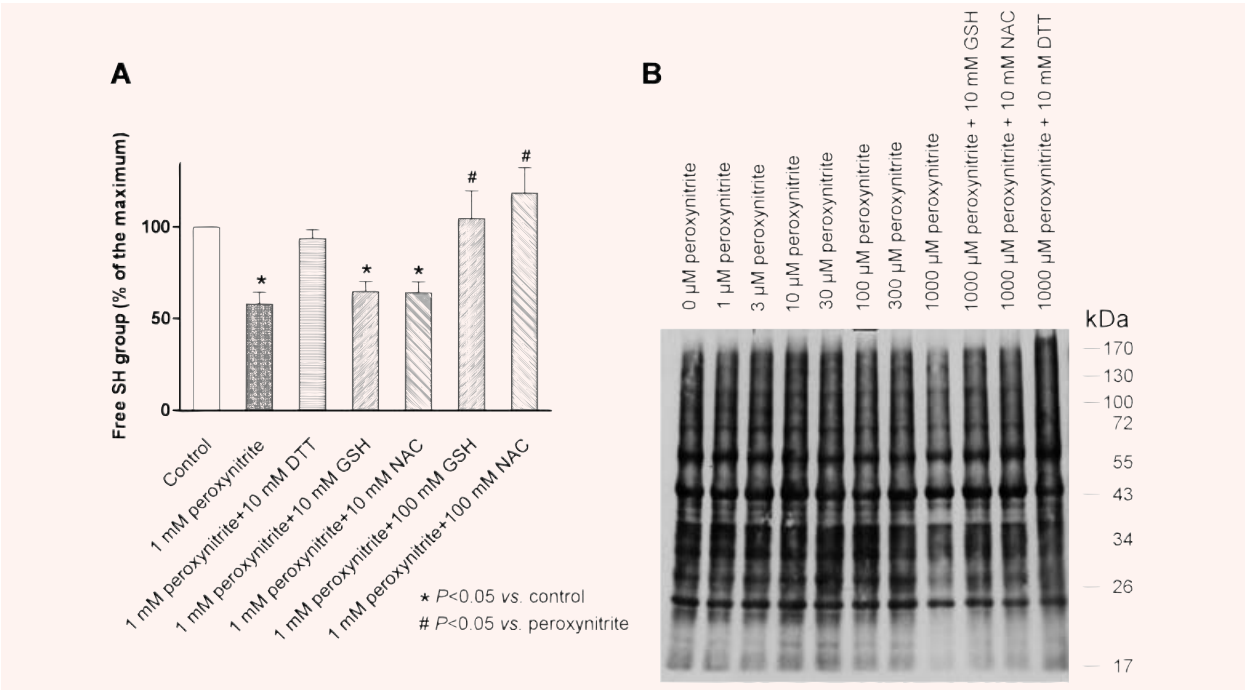

Fig. 3 Results of biochemical tests assessing myocardial protein SH-oxidation in response to 1-mM peroxynitrite exposures and subsequent applications of different reducing agents. (**A)** Bar graphs (means ± S.E.M.) illustrate the relative amounts of free (reduced) protein SH groups (untreated control: 100%) as resulted from the Ellman's assay. 1-mM peroxynitrite evoked a marked reduction in protein SH content, and 10-mM DTT, 100-mM GSH or 100-mM NAC gave rise to a full reversion in the total SH content after peroxynitrite. 10-mM GSH or 10-mM NAC were much less effective in reducing the peroxynitrite-oxidized SH groups than 10-mM DTT or 100-mM GSH or 100-mM NAC. Results are pooled from 9 independent Ellman's assays. (**B**) SH-oxidation of individual myocardial proteins. Identical amounts of myocardial proteins were subjected to SDS-PAGE following SH-specific biotinylation. Signal intensity of the immunoblotted proteins is proportional with the amount their free SH groups as revealed by the Western analysis. SH oxidation of myocardial proteins at all molecular weights was noticed following the application of 1-mM peroxynitrite. Subsequent application of 10-mM GSH, 10-mM NAC or 10-mM DTT gave similar results with the Ellman's assays. Assays of panel B were repeated 6 times.

SH oxidation is moderate but significant. Nevertheless, the SH oxidation-dependent component of peroxynitrite-evoked alterations in Ca^{2+}-regulated force production proved to be reversible.

Previous investigations pointed to the significance of protein SH groups in the maintenance of optimal intracellular redox environment and their involvement in cell defence against oxidative damage [20, 33–35]. In order to assure normal biological function, most cysteinil residues are maintained in a fully reduced state under physiological conditions [23, 36]. The reversible conversion of SH groups to disulfides is one of the earliest events during radical-mediated oxidation of proteins [37]. Myocardial protein SH content decreases characteristically during ischaemia and reperfusion, and SH oxidation of proteins may thereby contribute to the contractile dysfunction of the postischaemic stunned myocardium [20, 36, 38]. Some of the most important reactive species: the superoxide, H_2O_2 and peroxynitrite have been shown to directly cause SH oxidation in protein model systems and in cells [39, 40]. Our results with the SH-specific DTDP indicated that oxidation of myocardial SH groups has the potential to diminish contractile force. Collectively, these considerations underline the significance of SH oxidation of myocardial proteins, because marked oxidation of SH groups means not only the impairment of the first line of the antioxidant cell defence but also a potential danger for the contractile function.

In this study, we determined the SH oxidation-dependent component of peroxynitrite-evoked mechanical alterations in human cardiomyocytes and compared these effects with that of the SH-specific DTDP. 50-μM peroxynitrite decreased F_0 approximately to 50%. DTT or NAC increased F_0 significantly following 50-μM peroxynitrite, and hence identified an SH sensitive component of the peroxynitrite-induced mechanical dysfunction. A possible explanation for the finding that 10-mM NAC apparently did not reverse peroxynitrite-induced SH oxidation (Fig. 3) but it did reverse peroxynitrite-reduced F_0 values (Fig. 2B) may relate to a lower signal to noise ratio for the Ellman's assay than that for the mechanical measurements. Of note, 2.5-mM DTDP evoked a robust reduction in protein SH content and hence allowed the recognition of changes in the SH-oxidative status of proteins following 10-mM NAC or 10-mM GSH exposures (Fig. 4A). However, at lower levels of protein oxidation that occurred either after 1-mM peroxynitrite

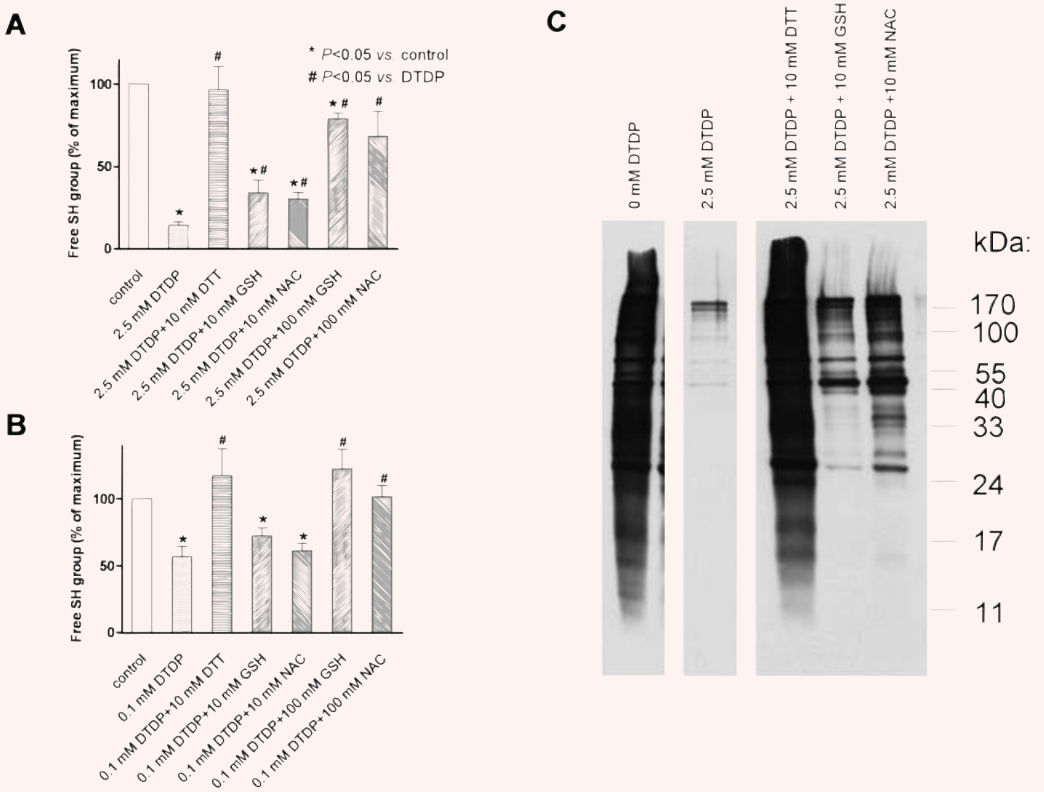

Fig. 4 Results of biochemical tests assessing myocardial protein SH-oxidation in response to DTDP exposures and subsequent applications of different reducing agents. (**A**) Bar graphs (means ± S.E.M.) illustrate the relative amounts of free (reduced) protein SH groups (untreated control: 100%) as resulted from the Ellman's assay. 2.5-mM DTDP evoked a robust reduction in protein SH content, and 10-mM DTT, 100-mM GSH or 100-mM NAC gave rise to reversion to a large degree in the total SH content after 2.5-mM DTDP. 10-mM GSH or 10-mM NAC were much less effective in reducing the DTDP-oxidized SH groups than 10-mM DTT, 100-mM GSH or 100-mM NAC. (**B**) 0.1-mM DTDP evoked comparable reduction in protein SH content to 1-mM peroxynitrite, and 10-mM DTT, 100-mM GSH or 100-mM NAC gave rise to full reversion in the total SH content after 0.1-mM DTDP. 10-mM GSH or 10-mM NAC were less effective in reducing the DTDP-oxidized SH groups than 10-mM DTT, 100-mM GSH or 100-mM NAC. Results of panel A and panel B are pooled from 4 independent Ellman's assays. (**C**) SH-oxidation of individual myocardial proteins as in Figure 3B. SH oxidation of myocardial proteins at selected molecular weights were noticed in a non-uniform fashion following the application of 2.5-mM DTDP. Subsequent application of 10-mM GSH, 10-mM NAC or 10-mM DTT gave similar results with the Elmman's assays. Assays of panel C were repeated 4 times.

(Fig. 3A) or after 0.1-mM DTDP (Fig. 4B), the increase in protein SH content did not reach significance either after 10-mM NAC or after 10-mM GSH, but only after the application of 100-mM NAC, 100-mM GSH or 10-mM DTT. Taken together, these results suggest that NAC and also GSH are capable to reverse peroxynitrite-induced SH oxidation, and that F_0 is more sensitive for this reversion than protein SH content as evidenced by our Ellman's assay.

When the SH-specific reducing agent DTT was applied after DTDP we observed full normalization in the SH content as well as in F_0 and $k_{tr,max}$. However, when DTT was applied after peroxynitrite, although it also reverted SH oxidation, it induced only a partial restoration in F_0. The full reversion of the free SH groups following DTDP or peroxynitrite illustrated DTT as a potent SH reducing agent, but it also pointed to the limited contribution of SH-oxidation to the mechanical dysfunction of peroxynitrite-treated cardiomyocytes.

Collectively, it appears that the mechanical alterations following peroxynitrite applications are attributable to a lesser degree to SH oxidation than to other types of protein alterations. The partial restoration in F_0 by DTT in cardiomyocytes treated with 50 μM peroxynitrite suggested that the contribution of SH-oxidation cannot be more than ~20% of the total reduction in F_0. Nevertheless, it should be pointed out that our assays were performed at room temperature and at more physiological temperatures the SH oxidation by peroxynitrite or DTDP can be different [41].

Results of earlier investigations from our laboratory demonstrated that the peroxynitrite-evoked force reduction is best paralleled by the nitration of a structural sarcomeric protein, α-actinin

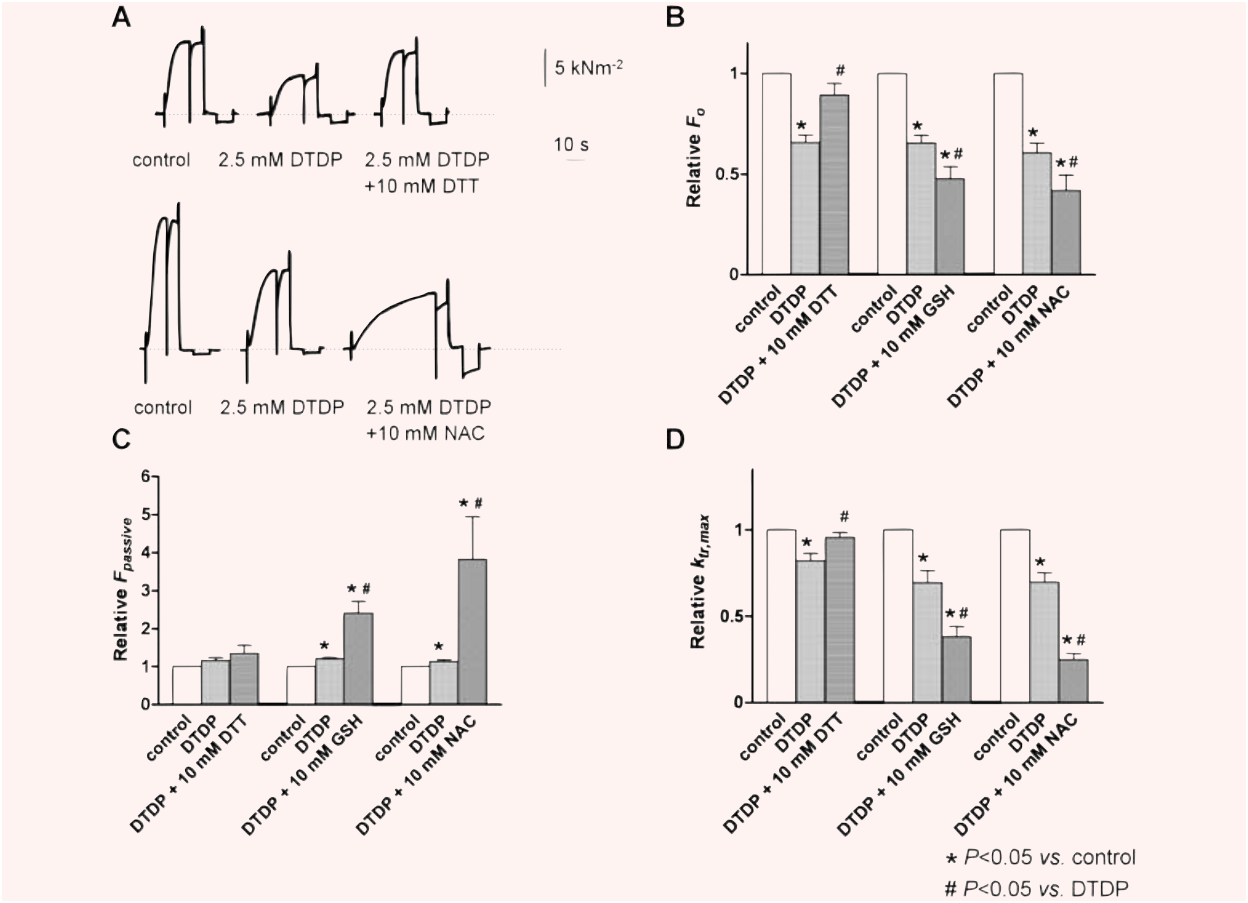

Fig. 5 Reversion of the mechanical effects evoked by 2.5-mM DTDP following the applications of SH-reducing agents. (**A**) Original recordings illustrate the mechanical effects of the sequentially applied 2.5-mM DTDP and 10-mM DTT (upper panel) and 2.5-mM DTDP and 10-mM NAC (lower panel). Bar graphs (means ± S.E.M.) illustrate the magnitudes of relative changes in F_o (**B**), $F_{passive}$ (**C**) and in $k_{tr,max}$ (**D**). DTDP decreased F_o, evoked a small increase in $F_{passive}$ and decreased $k_{tr,max}$. DTT induced an almost complete restoration in F_o and $k_{tr,max}$, and was without effects on $F_{passive}$. On the other hand, GSH or NAC induced a decreased F_o and $k_{tr,max}$ further, and evoked an increase in $F_{passive}$. The number of cardiomyocytes ranged between 8 and 26 for the different tests.

[22]. In contrast, the DTDP-evoked reduction in force was correlated with the oxidation of the sarcomeric actin and myosin light chain 1 [23]. The divergent involvement of sarcomeric protein modifications following peroxynitrite or DTDP applications may explain why cross-bridge kinetics was not altered following peroxynitrite exposures, while it was largely affected by DTDP. Our effort to identify a hypothetical group of proteins undergoing selective oxidation-reduction cycles by peroxynitrite and antioxidant exposures was precluded by the homogenous and the relatively small changes in signal intensities even at maximal peroxynitrite concentrations in our biotinylation assays. Hence, in this study we could not ascribe the peroxynitrite-evoked SH oxidation-dependent mechanical changes to one or another sarcomeric protein.

One of the salient aims of our present investigation was to compare the relative potentials of different reducing agents in reverting SH-dependent mechanical alterations in human cardiomyocytes. To this end, following peroxynitrite or DTDP administrations assays were performed with DTT, NAC and with the intracellular antioxidant GSH. In general, DTT possessed the highest potential in the reversion of the mechanical and biochemical effects. On the other hand, the results obtained with GSH and NAC were seemingly contradictious. After DTDP, both of these latter reducing agents induced a modest increase in the SH content. Interestingly, however these resulted in further diminution in F_o and in $k_{tr,max}$, and significant increases in $F_{passive}$. However, after peroxynitrite, NAC similarly to DTT evoked a partial reversion in F_o. Redox reactions in the cells are determined by many factors, among which are probably the most important the redox potential, the conformation and the molecular size of the reacting partners. Additionally, SH oxidizing agents can react in different ways with

the thiol groups of the proteins, generating intra- or intermolecular disulfide bridges or mixed disulfides. For example, DTDP besides generating disulfide bonds inside and among the proteins can also create a mixed disulfide between the cysteinil residues and one-half of the DTDP molecules, thereby liberating the other halves of DTDP molecules as thiopyridones [24]. DTT, due to its low redox potential, can reduce disulfides very rapidly and with a high efficiency seemingly irrespectively of the presence or absence of mixed disulfides [42]. Conversely, GSH and NAC, both having higher redox potentials than DTT, are less effective in reducing the different forms of oxidized SH groups. We assume, therefore, that these differences can contribute to the explanation of the complex interplay between oxidative and reducing agents included in our experiments. Although there are positive human studies with antioxidants, including NAC [43], the results of big clinical trials with antioxidants in preventing the initiation and progression of cardiovascular diseases are also variable [44–46]. The human clinical trials ended with negative outcomes and the results of this study emphasize the significance of the proper application and choice of antioxidants.

In conclusion, results of this model investigation revealed that the contribution of SH oxidation to the peroxynitrite-mediated contractile depression is inferior to other peroxynitrite-evoked biochemical effects in human cardiomyocytes. Our data also illustrated that Ca^{2+}-activated active force, Ca^{2+}-independent passive force and the kinetics of the actin-myosin cycle are in complex relations with myocardial protein oxidation. Different combinations of the reduced and oxidized myocardial proteins may exert opposing effects on these parameters. Hence, the extent of myocardial protein oxidation, and the molecular characteristics of the oxidoreductive insults should be also considered when the SH-dependent mechanical alterations are evaluated in human cardiomyocytes.

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