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ORIGINAL ARTICLE

Peroxynitrite-mediated oxidative modifications of myosin and implications on structure and function

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Abstract

The peroxynitrite-induced functional impairment of myosin was studied in different reaction conditions, known to alter the oxidative chemistry of peroxynitrite, to better understand the molecular mechanisms of this interaction. It is shown that peroxynitrite is able to enhance the basal MgATPase activity up to 2-fold while inhibiting the actin-stimulated ATPase activity of myosin and that the extent of these functional alterations is dependent on the reaction medium. The observed changes in the stimulation of the MgATPase activity correlate with the extent of carbonyl formation in myosin. The enzyme inhibition is more potent in conditions where the efficiency of tyrosine nitration and peroxynitrite reactivity towards sulphydryls are lower. Together with the observation that reversion of sulphydryl oxidation did not lead to the recovery of myosin functional and structural impairments, these results point out to the importance of protein carbonylation as a post-translational modification in the peroxynitrite-induced myosin functional impairment.

Keywords: *Myosin, peroxynitrite, protein oxidation, muscle contraction*

Abbreviations: *ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; DNPH, 2,4-dinitrophenylhydrazine; DSC, differential scanning calorimetry; DTNB, 5,5'-dithio-bis(2-nitrobenzoate); DTT, dithiothreitol;* IC_{50}*, concentration of inhibitor required to produce 50% inhibition; F-actin, polymerized form of monomeric actin; S1, myosin subfragment-1; SDS, sodium dodecyl sulphate; SIN-1, 3-morpholinosydnonimine; Tris, tris(hydroxymethyl)aminomethane hydrochloride.*

Introduction

Studies performed in the last decade have suggested that peroxynitrite ($ONOO^-$), a potent biological oxidizing agent formed in the diffusion-rate limited reaction between nitric oxide ($^{\bullet}NO$) and superoxide anion ($O_2^{\bullet-}$) [1,2] may be responsible, at least in part, for muscle dysfunctions observed in several pathophysiological conditions. This is supported by the detection of the chemically stable 3-nitrotyrosine (3-NT) produced by the reaction of peroxynitrite with protein tyrosines on skeletal and cardiac muscle proteins during ageing [3–5] or in numerous disease conditions including sepsis, acute lung injury, myocarditis, atrial fibrillation, doxorubicin cardiomyophathy and ischemia/reperfusion insults [6–10]. However, the relationships among high levels of 3-NT groups, oxidative stress and muscle dysfunctions remain uncertain. It is well known that tyrosine nitration may affect protein structure and function, nevertheless the relevance of the observed structural and functional alterations depend on the extent and location of the nitrated tyrosine residues within the protein. In skeletal and cardiac muscle tissue, some studies have pointed out a correlation of protein nitration with functional and structural modifications, for example nitration of Ca^{2+}-ATPase from sarcoplasmic reticulum with loss of Ca^{2+}-homeostasis [11,12], nitration of creatine kinase with impairment of myofibrillar energetics [8,10] or nitration of α-actinin with the deterioration

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of the myofibrillar cross-striated pattern and subsequent reduction of isometric force production [7].

Peroxynitrite can directly produce oxidation of protein cysteines to thiyl radicals ($RS^{\bullet}$), disulphides (RSSR) and eventually to sulphenic acid (RSOH) [13,14], although at physiological pH the two electron oxidation leads predominantly to disulphides [15]. In addition, peroxynitrite can modify proteins by acting through radicals generated during its spontaneous decomposition in biochemical buffers and biological fluids. At neutral pH $ONOO^-$ is partially protonated (ONOOH, pKa = 6.5–6.8). Whereas peroxynitrite anion is relatively stable, peroxynitrous acid decomposes rapidly to nitrate forming the hydroxyl radical ($HO^{\bullet}$) and nitrogen dioxide ($^{\bullet}NO_2$) as reaction intermediates [16]. Oxidation of amino acid side chains leading to the formation of protein carbonyls can be produced through this radical pathway [17]. Peroxynitrite anion can also react fast with carbon dioxide to yield nitrogen dioxide ($^{\bullet}NO_2$) and carbonate ($CO_3^{\bullet-}$) radicals [18,19]. Normally, 3-nitrotyrosine is a characteristic product of this reaction pathway in proteins.

Among myofibril proteins, myosin has been indicated as one of the proteins showing higher levels of 3-NT in some of the pathophysiological conditions cited above [3,5,8]. Myosin, a major component of myofibrils, is composed of two heavy chains, two essential light chains and two regulatory light chains. The globular head of myosin, named subfragment-1 (S1), contains both ATP and actin binding sites and is responsible, along with actin, for the conversion of ATP chemical energy into mechanical work during contractile activity. We have originally demonstrated that exposure of skeletal muscle purified myosin subfragment-1 (S1) to sub-micromolar fluxes of peroxynitrite produced by SIN-1 decomposition induces a strong inhibition of the actin-stimulated myosin-ATPase activity which correlated with structural modifications that decrease the thermal stability of S1 leading to a partially unfolded state [20]. Whereas some consensus appears to exist regarding myosin sensitivity to peroxynitrite-induced functional and structural impairment, the chemical oxidative modifications contributing to such alterations is yet controversial. A linear relation has recently been suggested between the degree of tyrosine nitration and functional deficits produced by $ONOO^-$ on cardiac myosin [21]. However, a direct relationship between the two events is difficult to demonstrate because conditions that favour protein nitration can lead to the simultaneous oxidation of other amino acids that might play crucial roles in modulating protein function. In addition, it is well known that the ability of peroxynitrite to modify certain amino acid residues can be influenced by many different factors such as the buffering system, pH or the presence of bicarbonate [22,23]. Therefore, our aim in the present study was to define more clearly the chemical modifications induced by peroxynitrite in skeletal muscle myosin that leads to a significant loss of functional activity. To this end, we assessed and compared the enzyme activity, the structural and the chemical alterations upon myosin subfragment-1 exposure to chemically synthesized peroxynitrite in different experimental reaction conditions known to alter the oxidative chemistry of peroxynitrite. By using the amine-based buffer Tris vs phosphate buffer in the absence or presence of bicarbonate we have modulated the different chemical reaction pathways of peroxynitrite with myosin leading to different extent of chemical modifications, such as cysteines oxidation, tyrosine nitration and protein carbonyls formation. In addition, to account for conformational effects on myosin structure and to better reproduce the cellular conditions under which myosin oxidation may occur *in vivo* the effects of myosin endogenous ligands on myosin modifications upon exposure to peroxynitrite have been examined, as well as the ability of disulphide bridge reducing agents to reverse the observed loss of structure and function. These studies provide new insights on the molecular mechanisms by which peroxynitrite may impair the actin-stimulated myosin ATPase activity and muscle contractility.

Materials and methods

Chemicals

Chemicals used to prepare buffers were reagent grade. ATP DNPH, DTNB, NADH, 3-nitrotyrosine, phosphoenolpyruvate and trypsin were supplied by Sigma Chemical Co (St. Louis, MO). Pyruvate kinase and lactate dehydrogenase were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Monoclonal *anti*-(3-nitrotyrosine) Ig was purchased from Calbiochem (catalogue number 487923, La Jolla, CA) and the secondary IgG conjugated with horseradish peroxidase was supplied by BioRad (Hercules, CA).

Preparation of myosin subfragment-1 and actin

Myosin and myosin subfragment-1 (S1) have been prepared as indicated previously [20]. Briefly, myosin was purified from leg and dorsal white rabbit skeletal muscle and S1 prepared by chymotryptic digestion of myosin as described elsewhere [24]. F-actin was prepared from acetone powder of rabbit skeletal muscle as previously described [25]. Protein concentrations were determined spectrophotometrically by using extinction coefficients of $E^{1\%}_{280} = 5.7\ cm^{-1}$ for myosin, $E^{1\%}_{280} = 7.5\ cm^{-1}$ for S1 and $E^{1\%}_{290} = 11.5\ cm^{-1}$ for actin. The molecular weights used were 500, 115 and 42 kDa for myosin, S1 and actin, respectively. Prior treatments with peroxynitrite, S1 and actin were dialysed against the chosen buffer solution which were free of reducing agents.

Peroxynitrite synthesis

Peroxynitrite was synthesized according to the method described elsewhere [13] and briefly outlined in a previous study [26]. Peroxynitrite solution was split into small aliquots and frozen at −80°C until use. Peroxynitrite concentration was measured spectrophotometrically in aliquots thawed immediately before its use in the experiments reported in this paper, using an extinction coefficient of 1670 M^{-1} cm^{-1} at 302 nm in 1.5 M NaOH [27].

Treatment of myosin with peroxynitrite

Myosin subfragment-1 was diluted to 1 mg protein/mL in 0.5 M Tris-HCl, pH 7.2 or 0.5 M K_2HPO_4/KH_2PO_4, pH 7.2 buffer solutions in the absence or presence of 25 mM potassium bicarbonate ($KHCO_3$) and peroxynitrite was added in a range of concentrations as a single bolus with simultaneous gentle vortex stirring. Thereafter, the solution was incubated at 25°C for 10 min to allow for complete reaction before carrying out chemical, structural or functional assay measurements. The amount of NaOH added to the samples was lower than 30 mM and the observed increase of pH was always lower than 0.25 units. In control experiments S1 was treated (a) with the same amount of NaOH solution (1.5 M) added to the samples with $ONOO^-$ and (b) with decomposed $ONOO^-$. In both cases, the effects were found to be negligible.

Measurements of 3-nitro-Tyr formation, Cys oxidation and protein carbonyls

The extent of 3-nitrotyrosine (3-NT) formation was measured spectrophotometrically, using extinction coefficient values at 415 nm of 3330 and 3056 M^{-1} cm^{-1} determined from measurements of pure 3-NT solutions prepared by weight in Tris and phosphate buffer solutions, respectively. In addition, nitration of tyrosines was also assessed by Western blot. Briefly, 12% polyacrylamide gels were loaded with 10 μg S1, electrophoresed for 2 h at 90 V in 25 mM Tris running buffer with 0.1% SDS and then transferred to nitrocellulose membranes. Prior to immunodetection, protein bands were visualized using the reversible protein staining solution Ponceau. After blocking, nitrocellulose membranes were incubated with anti-(3-NT) primary antibody (1:5000 dilution) and then with a goat anti-mouse-HRP conjugated secondary antibody (1:25 000). Colourimetric detection was performed with the Opti-4CN amplification kit from Bio-Rad. Quantification was carried out using the software Quantity One (Bio-Rad) by determining the average pixel intensity of each band.

Titration of S1 cysteines was done with DTNB, as previously indicated [20] using an extinction coefficient at 412 nm of 12 000 M^{-1} cm^{-1} for the coloured product thionitrophenolate. Cysteines were titrated with 0.5 mM DTNB and an S1 concentration of 0.5 mg protein/ml in 10 mM Tris and 25 mM KCl (pH 8.5). The increase in absorbance at 412 nm was continuously recorded during 10 min and then treated with 1% SDS, heated during 5 min at 80°C and the absorbance measured after cooling during 15–30 min until reaching a steady value.

Protein carbonyls formation was measured spectrophotometrically using DNPH as in Dalle-Donne et al. [28]. Briefly, the protein sample (1 mg/ml) was divided into two 0.5 ml aliquots, a Test and a Blank. The test aliquot was reacted with 0.5 ml of 10 mM DNPH in 2 M HCl while the Blank was reacted only with 2 M HCl for 1 h in the dark at room temperature and with vortexing every 10 min. The samples were precipitated with 10% TCA (final concentration) and centrifuged for 3 min at 11 000 g. The supernatants were discarded and the protein pellets washed three times with 1 ml portions of ethanol/ethyl acetate (1:1, v/v). The final pellets were resuspended in 1 ml of 6 M guanidine hydrochloride (dissolved in 20 mM phosphate buffer, pH 2.3) for 15 min with vortexing. Carbonyls content were estimated by the difference in absorbance at 366 nm (Test - Blank) using a molar absorption coefficient of 22 000 M^{-1} cm^{-1}.

Measurement of Mg^{2+}-ATPase activities

Steady-state assays of the basal Mg^{2+}-ATPase and the actin-stimulated Mg^{2+}-ATPase of myosin subfragment-1 were measured spectrophotometrically at 25°C using the coupled enzyme pyruvate kinase/lactate dehydrogenase assay [20] with the following reaction mixture: 20 mM Tris-HCl (pH 7.0), 2.5 mM $MgCl_2$, 2 mM ATP, 0.42 mM phosphoenolpyruvate, 0.375 mM NADH, 18 U of lactate dehydrogenase and 18 U of pyruvate kinase, as in Tiago et al. [20]. Actin stimulated S1 Mg^{2+}-ATPase activity was measured by supplementation of the previous assay medium with nearly 10-fold molar excess F-actin over S1, unless indicated otherwise 4.5 μM F-actin and 0.45 μM S1, and we obtained an average activity of 1.1 ± 0.1 μmoles product per min per mg of S1 ($n > 10$ S1 and F-actin preparations). Under these experimental conditions F-actin stimulated ~ 15-fold the basal S1 Mg^{2+}-ATPase activity, i.e. the basal S1 Mg^{2+}-ATPase activity was on average 0.075 ± 0.010 μmoles product per min per mg of S1.

Differential scanning calorimetry (DSC) measurements

DSC measurements were done as described in Tiago et al. [20]. Briefly, scanning calorimetry measurements were carried out using a differential scanning calorimeter, Micro-Cal MC-2, operated at a scanning rate of 60°C/h and under a nitrogen pressure of

1.5–2 kg/cm^2 during the scan. The analysis of the calorimetric data was carried out with the Origin^{TM} software developed by MicroCal (Northampton, MA). The curve fitting used Marquadt methods based on non-linear least-squares, improving the guesses for each parameter using an iterative process until there is no further improvement of the fit (minimum chi-square value).

Data analysis

The data plotted in the figures are the averages of the experiments performed with at least three different myosin preparations and each preparation done, at least, by duplicate (i.e. $n \geq 6$ for each data point). All results are expressed as mean values and the error bars shown are the standard deviations. Statistical significance of data was analysed wherever indicated by Student *t*-test. Differences were considered to be significant at $p < 0.05$. Lines drawn in the figures of inhibition of the actin-stimulated myosin S1 ATPase activities as a function of $ONOO^-$ concentration are the best non-linear least-squares two-parameter fit to the equation $V = V_0 - (I_{max}[ONOO^-]/IC_{50} + [ONOO^-])$, where I_{max} (the maximum inhibition) and IC_{50} are the two iterative parameters used for the non-linear least-squares fit, V_0 is the ATPase activity before treatment with $ONOO^-$ and V is the ATPase activity after treatment with each $ONOO^-$ concentration.

Results

Effect of different experimental conditions on peroxynitrite-mediated chemical modifications on S1

Tyrosine nitration. Myosin subfragment-1 was exposed to bolus doses of chemically synthesized $ONOO^-$ in potassium phosphate buffer (0.5 M, pH 7.2, 25°C) or Tris-HCl (0.5 M, pH 7.2, 25°C) and the extent of 3-nytrotyrosine (3-NT) formation quantified by monitoring the absorbance at 430 nm as indicated in the Materials and methods section. Reaction with synthetic peroxynitrite resulted in a concentration-dependent increase in 3-NT formation (Figure 1A) and the slopes yielded an efficiency of tyrosine nitration of 5.0 ($r^2 = 0.999$) and 1.3 ($r^2 = 0.995$) mol 3-NT/mM $ONOO^-$ administered for phosphate and Tris-HCl buffers, respectively. Supplementation with 25 mM bicarbonate enhanced significantly the efficiency of nitration in Tris-HCl (4.2 mol 3-NT/mM $ONOO^-$, $r^2 = 0.997$), but no significant changes were found in phosphate buffer (5.6 mol 3-NT/mM $ONOO^-$, $r^2 = 0.995$). Furthermore, incubation of S1 with 2.5 mM MgATP prior administration of $ONOO^-$ reduced to half the efficiency of nitration (2.4 mol 3-NT/mM $ONOO^-$, $r^2 = 0.987$) relative to the absence of substrate in phosphate buffer.

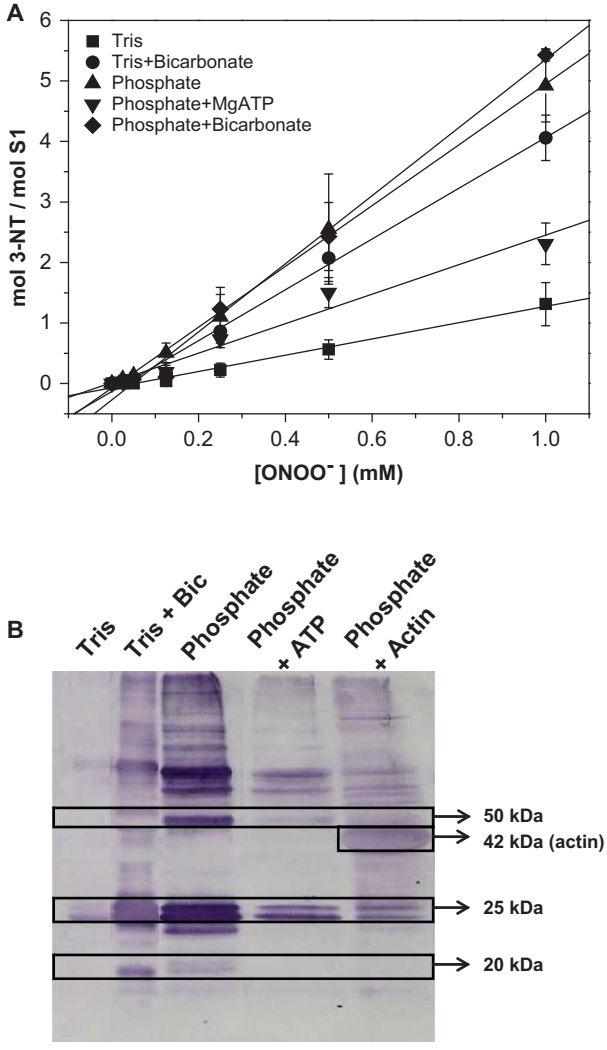

Figure 1. Nitration of S1 upon treatment with peroxynitrite in different experimental reaction conditions. (A) Spectrophotometric quantification of 3-nytrotyrosine (as indicated in Materials and methods) upon S1 exposure at 25°C to bolus doses (specified in the abscissa) of chemically synthesized peroxynitrite in potassium phosphate buffer (0.5 M, pH 7.2) and in Tris-HCl buffer (0.5 M, pH 7.2) either in the absence or presence of potassium bicarbonate (25 mM). For phosphate buffer it also shows the effect of MgATP (2.5 mM) during peroxynitrite treatment. Each data point represents the mean ± SD of at least three experiments performed in duplicate ($n \geq 6$). Phosphate and Phosphate$^+$Bicarbonate conditions were found to be not statistically significant by an analysis of variance test ($p > 0.05$). (B) Immunodetection of 3-nytrotyrosine (as indicated in Materials and methods) in tryptic S1 fragments upon S1 exposure to 0.25 mM peroxynitrite in the same conditions as before and additionally in phosphate buffer in the presence of actin. Rectangles indicate the position of the three fragments (50, 25 and 20 kDa) resultant from 30 min digestion with trypsin (molar ratio of 1:50 relative to S1).

To monitor in which protein domain nitration takes place, tryptic S1 fragments, derived from peroxynitrite-treated S1 in the different experimental conditions, were analysed by Western blotting. The results showed that nitration occurs primarily in the 25 kDa tryptic fragment (Figure 1B) being the relative extent of nitration in the different experimental conditions

consistent to the spectrophotometric quantification. It can also be observed in Figure 1B that the selectivity of Tyr nitration was not altered by the presence of ATP neither of F-actin during $ONOO^-$ treatment, despite that both myosin endogenous ligands afforded significant protection against nitration. It should also be noted that F-actin appears to be resistant to Tyr nitration under conditions where 3-NT formation is readily seen in myosin (as shown by the lack of significant labelling in the position of the 42-kDa band of actin).

Cysteines oxidation. Oxidation of highly reactive cysteines is one of the most established protein-chemical modifications induced by peroxynitrite and the catalytic subfragment-1 of myosin contains two highly reactive cysteine residues (Cys707 – SH1 and Cys697 – SH2) which are exposed at the water/protein interface of the 3D structure of myosin [29,30]. We have previously reported an extensive oxidation of myosin cysteines after treatment of S1 with SIN-1 [20]. In this work we have assessed whether the different experimental conditions which modulate the oxidative chemistry of peroxynitrite affect S1 sulphydryls reactivity. The oxidation of cysteines upon single bolus administration of peroxynitrite to S1 was confirmed by titration of the protein with DTNB. Fast reactive and total cysteines were assayed in the absence and presence of SDS, respectively, as indicated in the Materials and methods section. The total number of SH groups in S1 decreases from ~ 10 (in good agreement with the eight cysteines present in S1 heavy chain plus two cysteines present in the sequences of the two light chains, each containing one cysteine) to a maximum of 4.5 with the increase in peroxynitrite concentration (0.05–1 mM). The calculated IC_{50} values from curve fitting of the data, as indicated in the Materials and methods (not shown), are listed in Table I. The results indicate that peroxynitrite reactivity towards S1 fast reactive sulphydryls is essentially the same for phosphate and Tris buffer and it is slightly decreased in Tris buffer if bicarbonate is present (the IC_{50} value increases ~ 1.5-fold). On the other hand, when monitoring oxidation of the total number of cysteine residues the IC_{50} values vary depending on the buffer, i.e. it is 1.7-fold higher in phosphate than in Tris buffer and the inclusion of bicarbonate decreases further (1.7-fold) the IC_{50} value in Tris buffer. The addition of ATP during peroxynitrite treatments decreases the IC_{50} value ~ 1.4-fold with respect to phosphate buffer in the absence of ATP both for fast reactive and total cysteine residues.

Table I. Oxidation of S1 cysteines upon treatment with peroxynitrite in different experimental reaction conditions. Titration of cysteines was done as indicated in Materials and methods with 0.5 mM DTNB and 0.5 mg/ml S1 both in the absence and presence of SDS, for the fast reactive (very accessible) and total cysteines, respectively. Treatment with synthetic peroxynitrite (0.05–1 mM) produced a dose-dependent decrease of S1 cysteines from ~ 10 to a maximum of 4.5 with complete loss of the fast reactive cysteines and the curve fitting of the data provided the listed IC_{50} values for the different reaction conditions.

	IC_{50} (μM peroxynitrite)	
Experimental condition	Very accessible Cys	Total Cys
Tris	20.5 ± 5.2	74.2 ± 23.4
Tris + Bicarbonate	31.5 ± 5.1*	43.9 ± 7.4*
Phosphate	24.0 ± 2.0	124.2 ± 40.1*
Phosphate + MgATP	17.4 ± 2.8**	89.9 ± 26.1**

Data are means ± SD of at least three experiments performed in duplicate ($n \geq 6$).
*Significantly different from Tris ($p < 0.05$); **Significantly different from Phosphate ($p < 0.05$).

Protein carbonyls. The exposure of myosin to peroxynitrite has been previously reported to induce the formation of carbonyl groups as detected by western blotting [31]. By using the spectrophotometric DNPH assay we have determined the extent of carbonylation in peroxynitrite-treated S1 under different experimental conditions. The results, summarized in Figure 2, show that the higher carbonyl content is obtained when treatments are performed in phosphate buffer, where DNPH-reactive carbonyls increase from ~ 0.1 (unreacted protein) to a value close to 0.5 mol/mol of S1. The presence of bicarbonate during peroxynitrite treatments did not change significantly the levels of carbonylation in phosphate buffer (not shown), in good agreement with previous reports for the pH value used in this work [22], and it only slightly increased the levels in Tris buffer, from ~ 0.21 to 0.29 mol/mol S1 for the higher peroxynitrite concentration

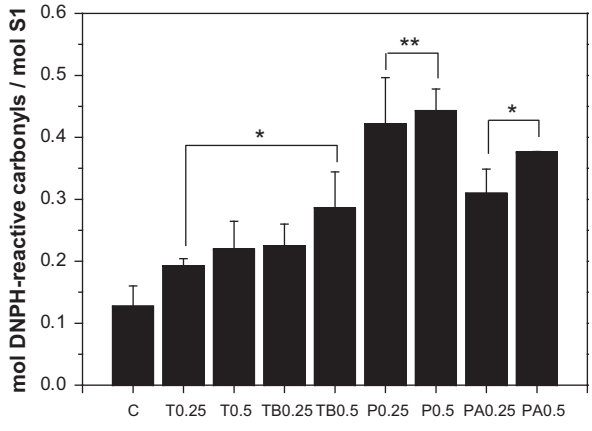

Figure 2. Carbonylation of S1 upon treatment with peroxynitrite in different experimental reaction conditions. Detection of carbonyls was performed in unreacted S1(C) and after S1 exposure to 0.25 or 0.5 mM peroxynitrite in the medium conditions indicated in the legend of Figure 1, i.e. Tris-HCl buffer (T), Tris-HCl buffer equilibrated with bicarbonate (TB), phosphate buffer (P) or phosphate buffer in the presence of MgATP (PA), by the reaction of the protein with DNPH followed by the spectrophotometric quantification of the acid hydrazones as indicated in Materials and methods. Values represent means ± SD of at least three experiments performed in duplicate ($n \geq 6$). *$p < 0.05$ compared to Phosphate buffer (P0.25 and P0.5) and unreacted S1; **$p < 0.05$ compared to all other medium conditions and unreacted S1.

(0.5 mM). On the other hand, the presence of ATP during peroxynitrite treatments in phosphate buffer decreases the carbonyl content ~ 1.4-fold for the lower peroxynitrite concentration (0.25 mM).

Effect of different experimental conditions on peroxynitrite-mediated functional impairment of S1

To determine the effects of exposure of S1 to peroxynitrite on myosin function, steady state assays of both basal S1 Mg^{2+}-ATPase and actin-stimulated S1 Mg^{2+}-ATPase activity were performed under the different experimental conditions mentioned above. It is known that chemical modifications of specific amino-acids in the S1 structure can result in changes in the ATPase cycle with either activation or inhibition of the basal Mg^{2+}-ATPase activity depending on the residues that are being modified [32–34]. Therefore, although the basal Mg^{2+}-ATPase activity, i.e. Mg^{2+}-ATPase activity measured in the absence of actin, does not correspond to the physiological activity related with myofibrils contraction it can give us some indications on the residues that may be modified upon exposure of myosin to peroxynitrite. In Figure 3A it can be observed that treatment of S1 with bolus doses of peroxynitrite induced an increase of the steady-state rate of the basal MgATP hydrolysis for all the reaction conditions except for phosphate buffer in the presence of ATP where a progressive inhibition above 0.5 mM peroxynitrite concentration was observed (Figure 3A). However, the relative increase in the Mg^{2+}-ATPase activity is considerably greater when treatments are performed in phosphate buffer (2-fold enhancement) than for the other conditions, namely Tris buffer in the absence (1.2-fold) or presence (1.3-fold) of bicarbonate. Furthermore, the activation of the Mg-ATPase activity showed a biphasic behaviour with maximum activation between 0.5–1 mM peroxynitrite concentration. On the other hand, the actin-stimulated Mg^{2+}-ATPase activity of S1 exposed to peroxynitrite was inhibited in a dose-dependent mode for all experimental conditions (Figure 3B) and the IC_{50} values derived from the best non-linear curve fit to the data are listed in Table II. As expected, the IC_{50} values for inhibition by synthetic peroxynitrite are much higher ($ONOO^-$/S1 molar ratio close to 20) than the ones previously obtained [20] for the inhibition by SIN-1 (SIN-1/S1 molar ratio of 5.5), likely because of peroxynitrite rapid decomposition when added as a single pulse. The results showed that the efficiency of inhibition of the actin-stimulated S1 ATPase activity was lower (1.5-fold) in Tris buffer with respect to phosphate buffer and was further decreased if bicarbonate is present during peroxynitrite treatments, i.e. the IC_{50} value increased 2.4-fold regarding Tris buffer in the absence of bicarbonate. Additionally, the efficiency of inhibition in phosphate buffer decreased nearly 2-fold when ATP is included during peroxynitrite treatments.

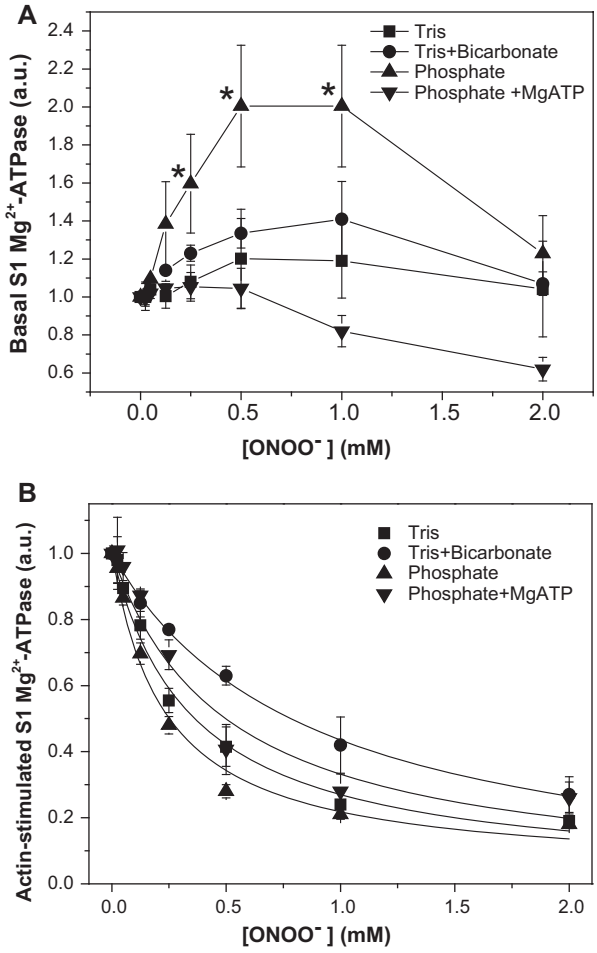

Figure 3. Functional impairment of S1 upon treatment with peroxynitrite in different experimental reaction conditions. S1 (1 mg/ml) was treated with bolus doses (specified in the abscissa) of chemically synthesized peroxynitrite in the medium conditions indicated in the legend of Figure 1 and after 2 h, the aliquots were pooled for assaying the activity as indicated in Materials and methods either in the absence (A) or presence (B) of F-actin in 10-fold molar excess with respect to S1. Each data point represents the mean ± SD of at least three experiments performed in duplicate ($n \geq 6$). $^*p < 0.001$ compared to all other medium conditions.

Reversibility of peroxynitrite-induced myosin oxidation by reducing agents

We have shown before that the major antioxidants present in normal muscle cells are able to significantly protect against inhibition of actin-stimulated S1 ATPase activity and S1 unfolding by SIN-1-derived peroxynitrite [20]. Nevertheless, the ability of some reductants to reverse loss of S1 function and structure has not been addressed before, in spite of the fact that cysteines oxidation is largely reversible *in vivo*. In order to better understand the relevance of cysteines oxidation in peroxynitrite-induced protein functional and structural alterations, reversibility studies were performed

Table II. Efficiency of inhibition of the actin-activated S1 Mg-ATPase activity upon treatment of S1 with peroxynitrite in different experimental reaction conditions. The IC_{50} values were derived from the best non-linear curve fit to the data drawn in Figure 3B as explained in Materials and methods.

Experimental condition	IC_{50} (μM peroxynitrite)
Tris	354.8 ± 52.6
Tris + Bicarbonate	863.5 ± 78.5*
Phosphate	234.8 ± 39.6*
Phosphate + ATP	430.1 ± 29.4**

Data are means ± SD of at least three experiments performed in duplicate ($n \geq 6$). *Significantly different from Tris ($p < 0.05$); **Significantly different from Phosphate ($p < 0.05$).

using dithiotreitol (DTT) as the reducing agent. Figure 4 illustrates that after treatment of S1 with 0.5 mM peroxynitrite, incubation with 1 mM DTT (2 h, 4°C) fully restored both fast reactive (Figure 4A) and total (Figure 4B) oxidized cysteines to its reduced state suggesting that peroxynitrite is inducing either inter- or intra-molecular reversible disulphide bridges. However, the reversion of cysteines oxidation was not paralleled by a recovery of the peroxynitrite-induced myosin functional impairment, as both basal and actin-stimulated S1 Mg^{2+}-ATPase activity were not altered after DTT post-treatment (Figure 5). Likewise, incubation with DTT failed to revert the loss of S1 thermal stability induced by peroxynitrite treatment. This can be observed in the DSC traces shown in Figure 6, which indicated a large decrease of the enthalpy of unfolding of S1 either with or without DTT incubation after treatment with peroxynitrite.

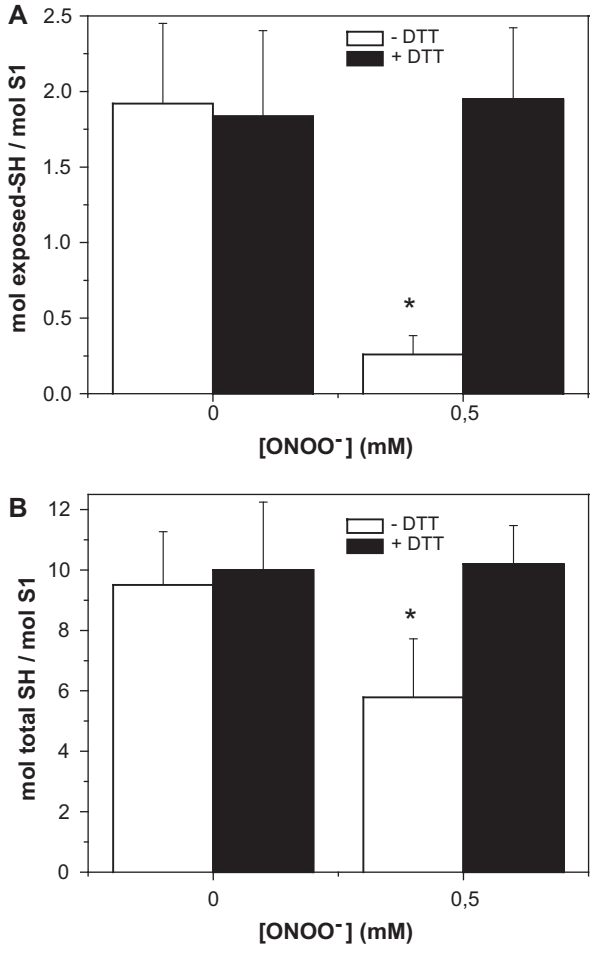

Figure 4. Reversion by DTT of peroxynitrite-induced S1 cysteines oxidation. Titration of fast reactive (A) and total (B) cysteines was performed as indicated in the legend of Table I on S1 before and after treatment in phosphate buffer (0.5 M, pH 7.2) at 25°C with peroxynitrite (0.5 mM) and post-treatment for 2 h at 4°C with DTT (1 mM). Prior titration with DTNB, S1 samples treated with DTT were dialysed against phosphate buffer in order to remove all the reducing agent that did not react. Values represent means ± SD of at least three experiments performed in duplicate ($n \geq 6$). Treated S1 is statistically significant from non-treated S1 and treated S1 with DTT post-treatment ($^*p < 0.001$).

Discussion

Oxidative post-translational modifications of myofibrilar proteins have been demonstrated to correlate with the development of various muscle dysfunctions suggesting that these modifications play important roles in regulating muscle function. Myosin, the most abundant myofibrillar protein, has been reported to be nitrated in ageing and some cardiac disease states. Studies about the effects of peroxynitrite on purified myofibril proteins are scarce and only a few reports have described the functional alterations of myosin upon exposure *in vitro* to peroxynitrite, which can be compared directly to our studies [20,21]. We have previously shown that exposure of purified myosin subfragment-1 to sub-micromolar fluxes of peroxynitrite produces extensive oxidation of cysteines, partial S1 unfolding and strong inhibition of the actin-stimulated myosin ATPase activity [20]. Although 3-NT formation could be detected, the extent of this chemical modification was found to be too low to account for the observed inhibition. On the contrary, in a recent report, Snook et al. [21] have observed no net loss of reactive cysteines in cardiac myosin upon exposure to peroxynitrite concentrations as high as 100 μM and concluded that the functional deficits inflicted by $ONOO^-$ were linearly related to the level of tyrosine nitration. Considering the elevated similarity (above 90%) between the two myosin isoforms (rabit skeletal vs rat cardiac) used in the referred studies, one possible interpretation for such different results could be the presence of myosin-binding protein C in the cardiac myosin preparations. In fact, the authors did not discard the hypothesis of oxidative modifications on this protein, contributing to the observed functional changes. Another possibility for the apparent discrepancy between the two studies could be the different experimental conditions used to expose myosin to peroxynitrite. Depending on the reaction medium conditions, peroxynitrite can react with different amino

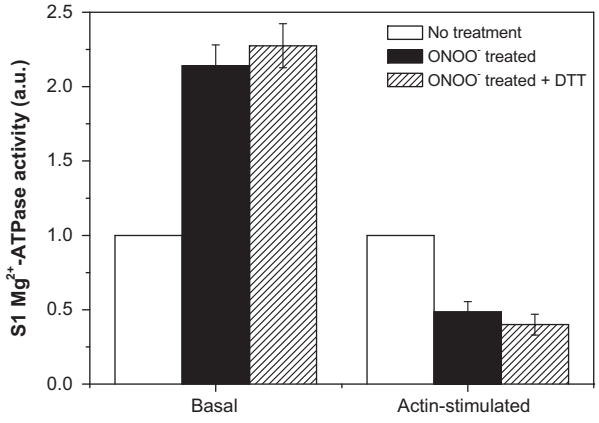

Figure 5. DTT failed to revert peroxynitrite-induced S1 functional impairment. S1 was treated with 0.5 mM peroxynitrite in phosphate buffer (0.5 M, pH 7.2) at 25°C and post-treated with 1 mM DTT for 2 h at 4°C. Aliquots of non-treated (activities taken as unity) and treated myosin-S1 samples (± DTT) were pooled for assaying the Mg^{2+}-ATPase activity as indicated in the legend of Figure 3 either in the absence (basal) or presence (actin-stimulated) of F-actin. Values represent means ± SD of at least three experiments performed in duplicate ($n \geq 6$).

acids and can form multiple products. The fact that in our previous report peroxynitrite was generated *in situ* by the $^{\cdot}NO$ and $O_2^{\cdot-}$ releasing compound SIN-1 may justify, in part, the low levels of nitration obtained. Although SIN-1 offers clear advantages over synthetic peroxynitrite because it allows mimicking a chronic and more efficient exposure to peroxynitrite, closer to the *in vivo* situation, its decomposition is highly pH-dependent, forcing some studies to be performed at pH values above the physiological conditions disfavouring 3-NT formation. Moreover, the nitrating potential of SIN-1 by itself is described to be much lower than that of authentic peroxynitrite due to competing reactions of tyrosil radicals with $^{\cdot}NO$ and $O_2^{\cdot-}$ as it was described elsewhere [35,36] and for this reason SIN-1 may not be the ideal tool to investigate the nitrating potential of peroxynitrite. Alternatively, the use of Tris buffer as the reaction medium could also attenuate tyrosine nitration. In fact, in this work we have demonstrated that the efficiency of S1 tyrosine nitration in Tris buffer is almost 4-fold lower than that obtained in phosphate buffer, but it can be increased nearly 3-fold if bicarbonate is present in the reaction medium (Figure 1A). This is consistent with the higher stability of peroxynitrite in phosphate buffer with respect to amine-based buffers on one hand [23] and with the reactive intermediates ($CO_3^{\cdot-}$ and $^{\cdot}NO_2$) formed in the reaction between HCO_3^-/CO_2 and peroxynitrite on the other hand [18,19]. Therefore, depending on the medium conditions the chemical modification of myosin by peroxynitrite may take place through one of the following chemical reaction pathways: (i) direct reaction with peroxynitrous acid or peroxynitrite; (ii) reaction with decomposition products of peroxynitrous acid ($HO^{\cdot}$ and $^{\cdot}NO_2$); or (iii) reaction with $CO_3^{\cdot-}$ and $^{\cdot}NO_2$ formed upon reaction of $ONOO^-$ with bicarbonate (HCO_3^-/CO_2).

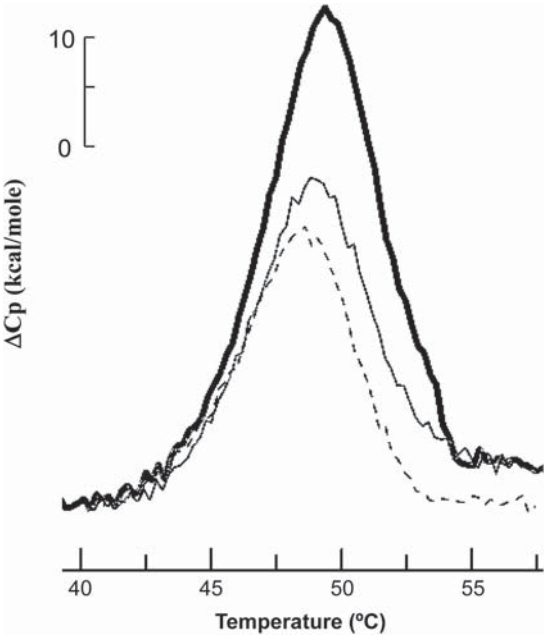

Figure 6. DTT failed to revert peroxynitrite-induced loss of S1 thermal stability. Representative DSC traces of S1 (2 mg/ml) non-treated (thick solid line) and treated with 0.25 mM peroxynitrite in phosphate buffer (0.5 M, pH 7.2) with (dashed line) or without (thin solid line) DTT (1 mM, 2 h, 4°C) post-treatment. Scans were performed at a rate of 60°C/h as indicated in Materials and methods.

Because CO_2 is a major end product of the aerobic catabolism in muscle, chemical modification of myosin through the (iii) reaction pathway should be favoured under oxidative stress conditions associated with heavy physical exercise and also in muscle acidosis elicited by inflammation. The presence of 25 mM bicarbonate is expected to inhibit the two electron thiol oxidation by peroxynitrite, because of CO_2 competition with the thiols for the direct reaction with this oxidant [18,37]. This was found to be the case for the peroxynitrite-induced oxidation of the two most reactive sulphydryls in myosin subfragment-1 as an increase of the IC_{50} values was observed in the presence of bicarbonate (Table I). However, the presence of bicarbonate was found to decrease the IC_{50} value for the total cysteine residues in S1 (Table I). These results indicate that oxidation of the fast and non-fast cysteine residues is occurring through a different reaction pathway. In this context it is important to recall here that thiols can also be oxidized by peroxynitrite-derived radicals by one-electron mechanisms to form thiyl, sulphinyl and disulphide radicals [38]. This is particularly relevant for the carbonate radical which is a strong one electron oxidant. Therefore, if non-accessible cysteines are preferentially oxidized by a one-electron mechanism it is expected that conditions favouring peroxynitrite secondary radicals, in particular $CO_3^{\cdot-}$, will yield lower IC_{50} values and, on

the contrary, conditions supporting a higher stability of the peroxynitrite anion will give higher IC_{50} values, as is observed for phosphate buffer.

Even though it is possible to redirect peroxynitrite reactivity towards nitration by alterations of the experimental reaction conditions, our results point out that 3-NT formation is always a minor modification with respect to the highly reactive cysteine residues oxidation on myosin. For example, in phosphate buffer when one cysteine is oxidized per S1 molecule approximately only one tyrosine is nitrated per 10 S1 molecules (calculated from the slope in Figure 1A and the IC_{50} value given in Table I). Moreover, pre-incubation with the myosin endogenous ligands ATP and F-actin decreased considerably the extent of 3-NT formation on S1 (Figure 1B). Analysis by Western blotting revealed that tyrosine nitration occurred preferentially in the 25 kDa tryptic fragment of S1 upon exposure in the different reaction mediums of the purified protein to peroxynitrite (Figure 1B). This location is consistent with the four nitrated tyrosines recently identified in the myosin heavy chain extracted from cardiac muscle of aged rats [5]. In fact, myosin nitration appears to be quite selective given that no differences were observed in nitration patterns between myosin in the presence and absence of its endogenous ligands ATP and F-actin. Whereas the protection afforded by F-actin can be simply attributed to competing reactions between peroxynitrite and the two target molecules, the effect of ATP is most likely related to alterations on the protein structure. As a nucleoside-containing molecule, ATP could be acting as a peroxynitrite scavenger [23], but the lack of protection provided by pre-incubation with ADP (results not shown) not only discards this hypothesis but reinforces a conformational effect. A major change in myosin subfragment-1 conformation is known to be associated with the ATP hydrolysis step [39]. During this process myosin goes from an open state similar to the structure of myosin in the presence of ADP to a closed state brought about by the formation of a transition state complex with the γ-phosphate. This is known to bring the two more accessible cysteines in a close proximity to form a disulphide bridge, which can explain the lower IC_{50} values obtained for the oxidation of cysteine residues when ATP is present in the reaction medium (Table I).

In order to clarify the reaction pathway of peroxynitrite with S1 leading to myosin functional impairment, we assessed and compared the effects of the different medium conditions on the S1 ATPase activity impairment produced by synthetic peroxynitrite. The first striking observation was that peroxynitrite is able to enhance the intrinsic Mg^{2+}-ATPase activity while inhibiting the actin-stimulated Mg^{2+}-ATPase activity of S1 and that the extent of activation and inhibition is dependent on the reaction medium (Figure 3). Previous studies have shown that chemical modifications of particular amino-acids in the myosin structure can result in changes of the ATPase cycle, leading to an increase of Pi dissociation rate so that the hydrolysis step becomes rate limiting and therefore the basal Mg^{2+}-ATPase activity is activated while the actin-activated Mg^{2+}-ATPase is inhibited [32–34]. The highly reactive Cys-707 (SH1) is one of the residues whose modification has been widely described to increase (up to 7-fold) the basal Mg-ATPase activity. Although we find an extensive oxidation of cysteine residues on S1 upon exposure to peroxynitrite (Table I and Figure 4) the possibility that SH1 oxidation is accounting for the observed activation of Mg-ATPase activity in the absence of actin has been discarded because of two main reasons. First, at peroxynitrite concentrations where the activation of the Mg-ATPase activity is maximum there are at least four cysteine residues already oxidized, including the two most reactive cysteine residues SH1 and SH2. In these regard it should be noted that only the selective modification of the SH1 group causes the enhancement of the basal Mg-ATPase activity as the further modification of the SH2 group results in inhibition of this activity. Second, the reversion of cysteines oxidation to its reduced state by post-incubation with 1 mM DTT failed to restore the basal Mg-ATPase activity (Figure 4).

An alternative residue on myosin whose chemical modification has been shown to enhance (up to 20-fold) the basal Mg-ATPase activity is Lys-84, the most reactive lysine residue (RLR) of the myosin head [40,41]. Lys-84, located in the 25 kDa NH_2-terminal tryptic fragment, resides at the interface with the 20 kDa tryptic fragment in a crevice that extends to the reactive cysteine SH1 [42,43]. Therefore, these two residues are in close proximity and lie at a domain interface that greatly influences the myosin ATPase cycle. While other oxidative modifications may also contribute to the myosin functional impairment, our data strongly support the hypothesis that peroxynitrite-induced oxidation of Lys-84, likely with resultant carbonyl derivatives, is playing a critical role in the observed ATPase cycle alterations. Indeed, results not shown indicate a reduction to about half (from ~ 1 to 0.5 mol/mol S1) of the maximal amount of trinitrophenylated RLRs when S1 is treated with 0.5 mM peroxynitrite, i.e. concentration where we found maximal stimulation of the basal Mg^{2+}-ATPase activity. Moreover, the observed changes in the stimulation of the basal Mg^{2+}-ATPase activity appear to correlate with the extent of carbonyl formation in S1 for the different experimental conditions in which the higher values are obtained for treatments performed in phosphate buffer followed by Tris buffer in the presence and then in the absence of bicarbonate (Figure 2). Furthermore, when treatments are performed in the presence of ATP, carbonyl formation was shown to be lower with respect to phosphate buffer (Figure 2) and in this condition no enhancement of the basal Mg^{2+}-ATPase is observed. This is in good agreement with previous reports

showing that the rate of chemical modification of Lys-84 in myosin is actually reduced in the presence of the substrate [42,44]. Based on carbonylation assays, myosin has been reported to be a particularly sensitive target in skeletal and cardiac muscles [31,45,46]. In addition, myosin heavy chain carbonylation has been correlated with a decreased myosin activity upon *in vitro* exposure of myosin to peroxynitrite [31].

Finally, the results of this work shed light on the mechanism of inhibition of the physiological actin-activated myosin Mg^{2+}-ATPase activity by peroxynitrite. First, the inclusion of bicarbonate in the reaction medium increased considerably tyrosine nitration in Tris buffer but has a partial protective effect against the enzyme inhibition (Table II), reinforcing the idea that nitration plays only a secondary role in the peroxynitrite-induced myosin functional impairment. Secondly, inhibition appears to be more efficient in conditions where peroxynitrite reactivity towards S1 sulphydryls is lower, i.e. phosphate $>$ Tris $\geq$ phosphate+ ATP $>$ Tris+bicarbonate (Table II). In addition, reversion of sulphydryl oxidation did not elicit the recovery of S1 functional (Figure 5) and structural (Figure 6) impairment. On these grounds, it merges the possibility that cysteine residues on the myosin structure are acting as scavengers of peroxynitrite or peroxynitrite-derived species, helping to prevent other oxidative modifications that may contribute significantly to the decline in myosin function *in vivo*.

In conclusion, our data suggest that protein carbonylation may be an important mechanism of peroxynitrite-induced myosin functional impairment in ageing and some muscle dysfunctions in which peroxynitrite is produced, such as ischemia-reperfusion, inflammation and strenuous or exhausting exercise. Our results do not exclude, however, a link between myosin unfolding, which seems to be an irreversible process even after reversal of sulphydryls oxidation, and oxidation of myosin cysteine residues. It is worth noting that irreversible post-translational modifications may accumulate *in vivo*, particularly for slow turnover proteins such as myosin, and are generally associated with a permanent loss of function.

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