

## Peroxynitrite induces F-actin depolymerization and blockade of myosin ATPase stimulation <sup>☆</sup>

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### Abstract

Treatment of F-actin with the peroxynitrite-releasing agent 3-morpholiniosydnonimine (SIN-1) produced a dose-dependent F-actin depolymerization. This is due to released peroxynitrite because it is not produced by ‘decomposed SIN-1’, and it is prevented by superoxide dismutase concentrations efficiently preventing peroxynitrite formation. F-actin depolymerization has been found to be very sensitive to peroxynitrite, as exposure to fluxes as low as 50–100 nM peroxynitrite leads to nearly 50% depolymerization in about 1 h. G-actin polymerization is also impaired by peroxynitrite although with nearly 2-fold lower sensitivity. Exposure of F-actin to submicromolar fluxes of peroxynitrite produced cysteine oxidation and also a blockade of the ability of actin to stimulate myosin ATPase activity. Our results suggest that an imbalance of the F-actin/G-actin equilibrium can account for the observed structural and functional impairment of myofibrils under the peroxynitrite-mediated oxidative stress reported for some pathophysiological conditions.

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Exposure of muscle cells to chronic oxidative stress conditions results in impairment of muscle contraction, which has been proposed to be, at least in part, the result of oxidative modification of myofibril proteins [1,2]. On the other hand, it has been shown that Cys<sub>374</sub> of actin monomer (G-actin) is particularly sensitive to oxidation by hydrogen peroxide [3] and nitric oxide donors [4], which are stress conditions leading to a reduction of the actin polymer (F-actin), that plays a major role in myofibril contraction. Peroxynitrite is an important oxidative stress agent in ischemia/reperfusion insults, such as heart infarct or atrial fibrillation [1,5,6], and inflammation [7], and it has been

reported that peroxynitrite can inhibit actin polymerization in neutrophils [8]. Furthermore, in aged muscle, actin is one of the proteins showing higher content of the commonly used fingerprint marker of peroxynitrite-reactive proteins 3-nitrotyrosine [9]. During inflammation and ischemia/reperfusion episodes tissue cells are exposed to fluxes of peroxynitrite ranging from submicromolar to micromolar concentrations [7,10]. SIN-1 is being used to mimic the effects of chronic exposure of cells in culture to a peroxynitrite oxidative stress [11,12], because it slowly decomposes in neutral and weakly alkaline aqueous solutions releasing nitric oxide and superoxide anion [13], which react with each other to produce peroxynitrite with a second order rate constant of  $(4-7) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$  close to the diffusion limit for chemical reactions [10,14]. Moreover, the kinetics of peroxynitrite generation and the peroxynitrite concentration attained in the solution during SIN-1 decomposition can be reliably monitored in the buffered solutions commonly used for biochemical studies with isolated subcellular components [15].

<sup>☆</sup> *Abbreviations:* DSC, differential scanning calorimetry; DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5'-dithio-bis(2-nitrobenzoate); IC<sub>50</sub>, inhibitor concentration required to produce 50% inhibition; S1, myosin subfragment 1; SIN-1, 3-morpholiniosydnonimine; SOD, superoxide dismutase; Tris, tris(hydroxymethyl)aminomethane hydrochloride; U, amount of enzyme that releases 1 μmol product per minute.

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In this work, we report that exposure to submicromolar fluxes of peroxynitrite, during 2 h, produces an extensive F-actin depolymerization that leads to an almost complete inhibition of myosin ATPase activity stimulation.

## Materials and methods

**Protein purification.** Actin was extracted from acetone powder of rabbit skeletal muscle in buffer G [2 mM Tris, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl<sub>2</sub>, 0.5 mM β-mercaptoethanol, and 0.005% NaN<sub>3</sub>] as described by Pardee and Spudich [16]. G-actin (M<sub>r</sub> 42.3 kDa) concentration was determined by measuring the absorbance at 290 nm using an extinction coefficient of 0.617 mg<sup>-1</sup> mL cm<sup>-1</sup> [17]. Myosin subfragment-1 (S1) has been prepared as indicated in Tiago et al. [18]. S1 (M<sub>r</sub> 115 kDa) concentration was determined spectrophotometrically by using an extinction coefficient of  $E_{280}^{1\%} = 7.5 \text{ cm}^{-1}$ .

**SIN-1 treatments on actin.** Actin samples (2 μM) were incubated with various concentrations of SIN-1 at 25 °C for 2 h, a time in which more than 95% of peroxynitrite derived from SIN-1 decomposition had already been released [15], in 10 mM Tris (pH 8.5), 25 mM KCl for F-actin and in 10 mM Tris (pH 8.5), 0.2 mM ATP, and 0.2 mM CaCl<sub>2</sub> for G-actin.

**Light scattering measurements.** F-actin depolymerization and G-actin polymerization were followed at 25 °C by changes in the light scattering intensity monitored at 90° with respect to the incident light, with both monochromators set at 546 nm [19], using a Perkin Elmer 650-40 spectrofluorimeter. To minimize error noise in a second series of experiments, the monochromators were set at 500 nm (excitation) and 510 nm (emission), and after intensity normalization both approaches gave the same results for F-actin depolymerization.

**Measurement of F-actin stimulated S1 Mg<sup>2+</sup>-ATPase activity.** The stimulation of myosin S1 Mg<sup>2+</sup>-ATPase activity by F-actin was measured spectrophotometrically at 25 °C in the buffer of SIN-1 treatment using the coupled enzyme pyruvate kinase/lactate dehydrogenase (0.42 mM phosphoenolpyruvate, 0.375 mM NADH, 18 U of lactate dehydrogenase, and 18 U of pyruvate kinase), as in Tiago et al. [18].

**Measurements of cysteine oxidation, 3-nitrotyrosine formation, and protein carbonyls.** The titration of actin cysteines was done with DTNB, as indicated in previous works [20,21], using an extinction coefficient at 412 nm of 13,200 M<sup>-1</sup> cm<sup>-1</sup> for the coloured product thionitrophenolate. The extent of nitrotyrosine formation was measured spectrophotometrically, using an extinction coefficient at 415 nm of 4560 and 4320 M<sup>-1</sup> cm<sup>-1</sup>, determined from measurements of pure 3-nitrotyrosine solutions prepared by weight in the buffer used for the treatment with SIN-1 of F-actin and G-actin, respectively. Protein carbonyl formation was measured spectrophotometrically using DNPH as in Dalle-Donne et al. [22]. Carbonyl content in actin samples was estimated by measuring the absorbance at 366 nm using a molar absorption coefficient of 22,000 M<sup>-1</sup> cm<sup>-1</sup>.

**Differential scanning calorimetry measurements.** DSC measurements were done as described in Merino and Gutiérrez-Merino [23]. Briefly, scanning calorimetry measurements were carried out using a differential scanning calorimeter MicroCal MC-2, operated at a scanning rate of 60 °C h<sup>-1</sup> and under a nitrogen pressure of 1.5–2 kg cm<sup>-2</sup> during the scan. To minimize the pH change during the temperature scan, due to the large enthalpy of protonation of Tris, the buffer used for F-actin treatments with SIN-1 was supplemented with 50 mM phosphate (pH 7) before loading the samples in the DSC. The analysis of the calorimetric data was carried out with the Origin™ software developed by MicroCal (Northampton, MA, USA). The curve fitting used Marquadt methods based on non-linear least-squares, being improved the guesses for each parameter using an iterative process until there is no further improvement of the fit (minimum  $\chi^2$  value).

**Chemicals.** Chemicals used to prepare buffers were reagent grade. ATP, ADP, DNPH, DTNB, NADH, 3-nitrotyrosine, phosphoenolpyruvate, and SIN-1 were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Pyruvate kinase and lactate dehydrogenase were purchased from Roche Molecular Biochemicals (Mannheim, Germany). ‘Decomposed

SIN-1’ was prepared leaving a solution of SIN-1 (1–10 mM) in buffer 10 mM Tris and 25 mM KCl (pH 8.5) to stand at room temperature for 12 h.

## Results and discussion

### *Exposure to submicromolar peroxynitrite generated during SIN-1 decomposition promotes F-actin depolymerization and inhibits F-actin stimulated myosin S1-ATPase activity*

Treatment of F-actin with SIN-1 resulted in a strong stimulation of the kinetics of F-actin depolymerization (Fig. 1A). As the intensity of light scattering of F-actin solutions was found to be linearly dependent upon F-actin concentration up to 9 μM F-actin (data not shown), the change of light scattering intensity can be converted in percent of polymerized F-actin taking into account the intensity signal of G-actin (Fig. 1A). The extent of F-actin depolymerization after complete decomposition of SIN-1 (i.e., after 2 h exposure to SIN-1) is dependent upon SIN-1 concentration (Fig. 1B), yielding an IC<sub>50</sub> value of 4.5 ± 0.5 μM SIN-1, i.e., approximately two moles SIN-1/mole of F-actin monomer. In addition, the onset of the kinetics of F-actin depolymerization is shortened as the SIN-1 concentration rises, due to the stimulation of the maximum rate of F-actin depolymerization, as shown by the increase of the maximum slope of the light scattering decay traces in Fig. 1A. The effects of SIN-1 on F-actin depolymerization are due to the short-lived peroxynitrite produced during SIN-1 decomposition, because: (i) they are not produced by ‘decomposed SIN-1’, (ii) SOD, which efficiently scavenges O<sub>2</sub><sup>-</sup> before reacting with NO<sup>+</sup> [12,15], and SOD plus catalase (to remove H<sub>2</sub>O<sub>2</sub> released by SOD activity) blocked the effect of exposure to SIN-1 on F-actin depolymerization (Fig. 1C), and (iii) hydrogen peroxide produced during SIN-1 decomposition in the buffer used in these experiments was lower than 0.5 μM and catalase did not prevent against the depolymerization of F-actin by exposure to SIN-1 (results not shown). From the kinetics of peroxynitrite production during SIN-1 decomposition under these experimental conditions [15,24] it follows that: (1) exposure of F-actin to peroxynitrite fluxes as low as 50–100 nM during 1–2 h produced nearly 50% F-actin depolymerization, and (2) the IC<sub>50</sub> for F-actin depolymerization takes place at a molar ratio of peroxynitrite/actin monomer close to 2.

Moreover, the extent of F-actin depolymerization induced by peroxynitrite is sufficient to produce blockade of myosin S1 Mg<sup>2+</sup>-ATPase activity stimulation by F-actin with an IC<sub>50</sub> of 20 ± 2 μM SIN-1 (Fig. 2), thus, pointing out that F-actin depolymerization induced by peroxynitrite can inhibit myofibril actomyosin ATPase activity. Therefore, exposure to peroxynitrite is eventually impairing G–F actin equilibrium inducing shortening of the actin filaments to a size smaller than that needed to activate myosin S1 ATPase.

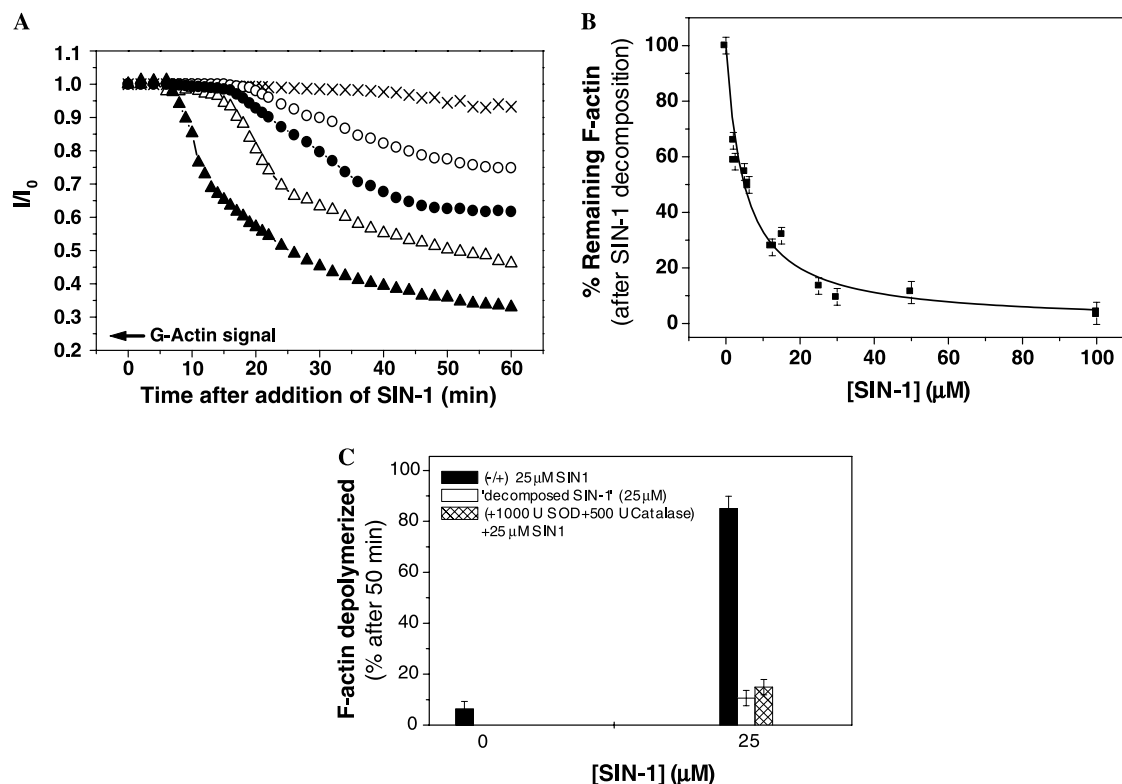


Fig. 1. F-actin depolymerization by peroxynitrite produced during SIN-1 decomposition. Measurements were done as indicated in Materials and methods, with 2  $\mu\text{M}$  F-actin. (A) Kinetics of F-actin depolymerization monitored by the decrease of light scattering intensity. Time dependence of the light scattering intensity relative to the intensity at time zero ( $I/I_0$ ) after addition of the following SIN-1 concentrations (in  $\mu\text{M}$ ): 0 (control, crosses), 2 (open circles), 6.25 (solid circles), 12.5 (open up-triangles), and 25 (solid up-triangles). The intensity signal for G-actin is indicated with an arrow. (B) Dependence upon SIN-1 concentration of the remaining F-actin 2 h after addition of SIN-1 (i.e., after complete SIN-1 decomposition), calculated from the light scattering intensity readings as indicated in the text. Data (solid squares) are plotted as means  $\pm$  SD. (C) F-actin depolymerization is not induced by 'decomposed SIN-1' or by treatment with SIN-1 in the presence of SOD (1000 U) plus catalase (500 U). Extent of F-actin depolymerization 50 min after addition of 25  $\mu\text{M}$  SIN-1 (black bars), 25  $\mu\text{M}$  'decomposed SIN-1' (white bars), and 25  $\mu\text{M}$  SIN-1 in the presence of SOD plus catalase (stripped bars). The results shown are the average of triplicate experiment.

*Differential scanning calorimetry pointed out that peroxynitrite-induced depolymerization correlated with a structural actin modification that lowers the cooperativity of the unfolding process and also protein thermal stability*

F-actin depolymerization by peroxynitrite released upon SIN-1 decomposition can also be monitored by DSC from the decrease of the peak height of the excess heat capacity at the critical unfolding temperature (Fig. 3A), which monitors the decrease of the cooperativity unit of the unfolding process. The F-actin concentration was raised up to 17.2  $\mu\text{M}$  to get low noise DSC traces. Again, neither 'decomposed SIN-1' nor treatment with SIN-1 in the presence of SOD plus catalase elicits a significant depolymerization of F-actin. In addition, DSC unraveled that F-actin depolymerization was accompanied by a parallel and large decrease of the thermal stability of F-actin (Figs. 3B and C), with the same  $\text{IC}_{50}$  value of  $36 \pm 4 \mu\text{M}$  for both processes, which correspond to a peroxynitrite to an actin molar ratio of  $2.1 \pm 0.2$ , a result very consistent with the result obtained in F-actin depolymerization measurements by light scattering.

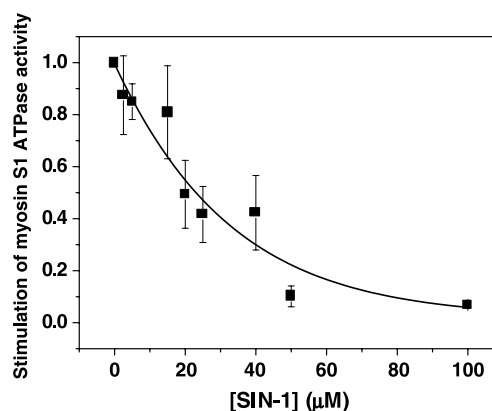


Fig. 2. F-actin treatment with SIN-1 inhibits actin stimulation of S1-myosin ATPase activity. Activity assays were done with F-actin treated for 2 h with the concentrations of SIN-1 indicated in the abscissa as indicated in Materials and methods. Data (solid squares) are plotted as means  $\pm$  SD. The results shown are the average of triplicate experiment.

*G-actin polymerization is also impaired upon exposure to submicromolar peroxynitrite fluxes*

DSC results pointed out that peroxynitrite-induced F-actin depolymerization is due to the peroxynitrite-

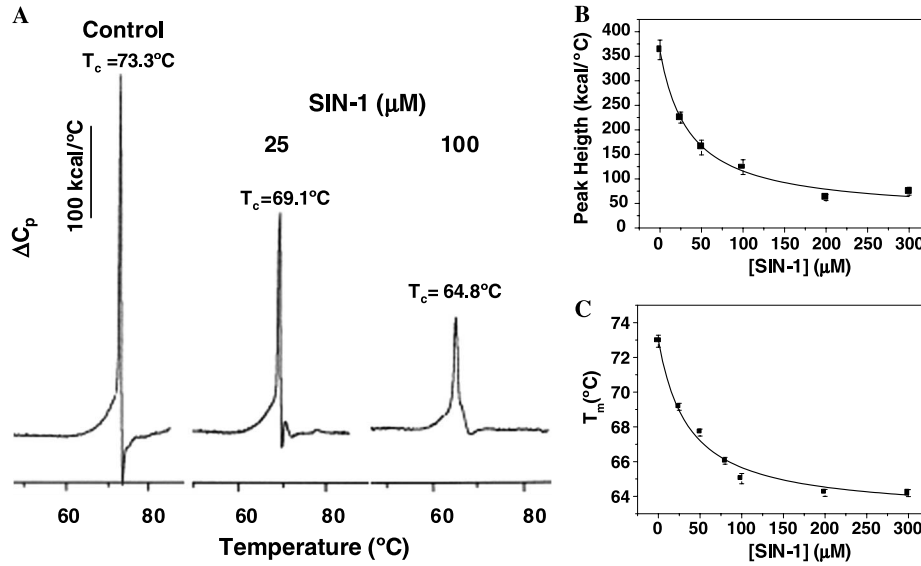


Fig. 3. Treatment with SIN-1 produced decrease of unfolding cooperativity and enhanced thermal instability of F-actin. DSC experiments were carried out as indicated in Materials and methods, in buffer: 50 mM Pi, 10 mM Tris, and 25 mM KCl (pH 7.35). The traces are representative of experiments done by triplicate with, at least, two different F-actin preparations. (A) DSC traces of F-actin (17.2  $\mu\text{M}$ ) pre-incubated for 2 h in buffer 10 mM Tris (pH 8.5), 25 mM KCl in the absence (control) or presence of 25 and 100  $\mu\text{M}$  SIN-1 before loaded in the DSC. (B) Dependence of the peak value of excess heat capacity of the unfolding of F-actin treated with SIN-1 upon the concentration of SIN-1. Data (solid squares) are plotted as means  $\pm$  SD. (C) Dependence of the critical unfolding temperature ( $T_c$ ) of F-actin treated with SIN-1 upon the concentration of SIN-1. Data (solid squares) are plotted as means  $\pm$  SD.

induced structural change on actin molecules. As within cells the level of F-actin is a balance between actin polymerization and depolymerization [4], we measured the effect of G-actin treatment with SIN-1 on the ability of G-actin to form F-actin. The results are shown in Fig. 4 and led to the conclusion that G-actin polymerization is inhibited by SIN-1 with an  $\text{IC}_{50}$  value of  $10 \pm 1$  and  $29 \pm 3 \mu\text{M}$  for the maximum rate and extent of G-actin polymerization, respectively.

*Exposure of actin to submicromolar peroxynitrite fluxes produced cysteine oxidation, but not detectable rise of protein carbonyls or of 3-nitrotyrosines*

Titration of actin cysteines with DTNB after 2 h exposure to different SIN-1 concentrations showed a dose-dependent decay of fast-reacting (exposed cysteines) and total actin cysteines (Fig. 5). SIN-1 induced oxidation of actin cysteines and this was also due to released

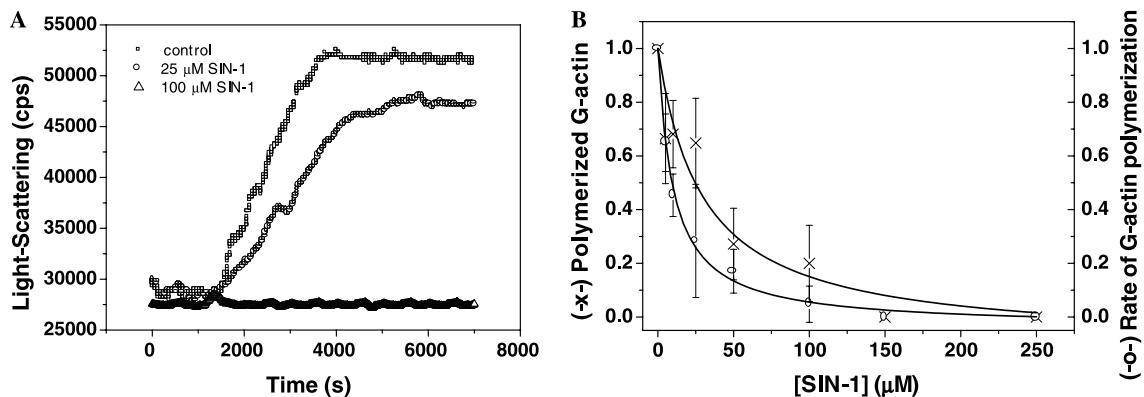


Fig. 4. Treatment with SIN-1 inhibited G-actin polymerization. G-actin (2  $\mu\text{M}$ ) polymerization was induced by 100 mM KCl and 2 mM  $\text{MgCl}_2$  at  $25^\circ\text{C}$ . (A) Kinetics of G-actin polymerization in the absence of SIN-1 (open squares) and in the presence of 25  $\mu\text{M}$  (open circles) and 100  $\mu\text{M}$  SIN-1 (open up-triangles) added to the buffer just before starting the polymerization. (B) Dependence upon SIN-1 concentration of the extent and maximum rate of G-actin polymerization relative to that in untreated G-actin (control). Symbols meaning: (x) extent of G-actin polymerization after saturation of kinetic traces like those shown in (A); (o) maximum rate of G-actin polymerization obtained from the maximum slope of kinetic traces. The results shown are the average of triplicate experiment.

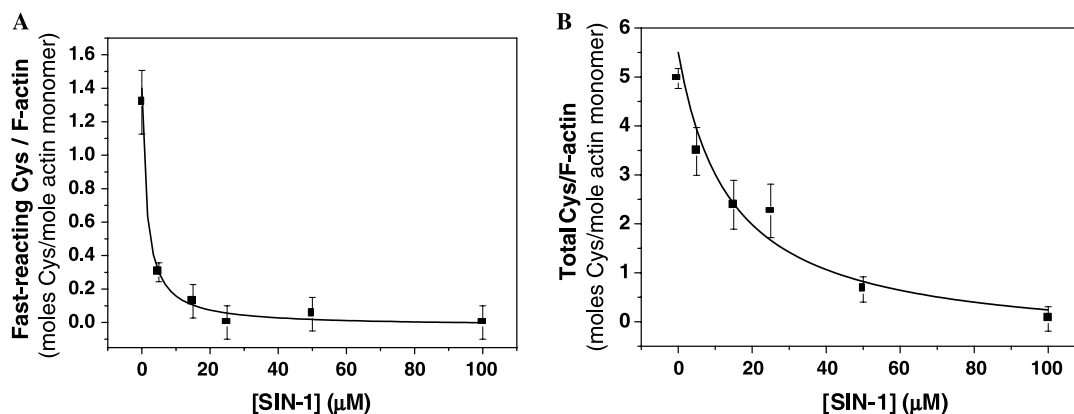


Fig. 5. F-actin cysteine oxidation upon treatment of F-actin with SIN-1. Titration of cysteines was done with 0.1 mM DTNB and 2 μM F-actin in 10 mM Tris and 25 mM KCl (pH 8.5). The increase in absorbance at 412 nm was continuously recorded during 10 min, and to measure total cysteines the samples were treated afterwards with 1% SDS, heated during 5 min at 80 °C and the absorbance was measured after cooling during 15–30 min until reaching a steady value. Cysteines per mole of F-actin monomer were calculated as indicated in Materials and methods. (A) Treatment with SIN-1 produced a dose-dependent decrease of F-actin exposed cysteines (i.e., cysteines reacting with DTNB in less than 1 min). Data (solid squares) are plotted as means ± SD. (B) Treatment with SIN-1 produced a dose-dependent decrease of total F-actin cysteines. Data (solid squares) are plotted as means ± SD. The results shown are the average of triplicate experiment.

peroxynitrite, because neither ‘decomposed SIN-1’ nor treatment with SIN-1 in the presence of SOD plus catalase produced significant actin cysteine oxidation, i.e., less than 0.2 cysteines/mole of actin. The IC<sub>50</sub> values for SIN-1-induced oxidation of exposed and total F-actin cysteines were 1.5 ± 0.5 and 14 ± 5 μM SIN-1, respectively. Bicarbonate (25 mM) did not enhance the effect of SIN-1, therefore, suggesting that peroxynitrite is promoting cysteine oxidation as a directly acting oxidant not through peroxynitrite-derived oxidant radicals, a reaction pathway which is favoured by bicarbonate [25]. This is further supported by the fact that after 2 h treatment with 10–100 μM SIN-1 concentrations the levels of protein modifications used as markers of oxidation by peroxynitrite-derived radicals, like 3-nitrotyrosines or protein carbonyls, are not detectable. As it has been shown that actin cysteine oxidation can contribute to the inhibition of actin polymerization as well as to the disruption of actin filaments [3,4,26], these results allow us to rationalize on simple grounds the depolymerization of F-actin induced by peroxynitrite.

## Conclusion

Exposure of F-actin and G-actin to peroxynitrite fluxes of 50–100 nM for 2 h is sufficient to promote nearly 50% F-actin depolymerization and 50% inhibition of the rate of G-actin polymerization, and peroxynitrite-induced F-actin depolymerization can efficiently block the stimulation of myosin ATPase by actin. These results pointed out that F-actin is between 10- and 20-fold more sensitive to peroxynitrite than other proteins of relevance to muscle bioenergetics reported so far, like creatine kinase [27], myosin [24], and sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase [21], and point out that actin filaments can be seen as a primary target in the impairment of muscle contraction by oxidative insults promoting a rise of peroxynitrite.

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