T. Tiago[1,2], D. Silva[1], A. Santos[1], M. Aureliano[1] and C. Gutiérrez-Merino[2]

[1]CCMar, Depto. de Química e Bioquímica, FCT, Universidade do Algarve, 8000-139 Faro, Portugal;
[2]Depto. Bioquímica y Biologia Molecular, Facultad de Ciencias, Universidad de Extremadura, 06071-Badajoz, Spain

Summary

In the present work we address the oxidative modifications accounting for the structural and functional impairment of the actomyosin complex under the oxidative stress mediated by peroxynitrite ($ONOO^-$). Experiments on purified myosin and actin have shown that submicromolar $ONOO^-$ concentrations produce strong inhibition of the F-actin stimulated myosin ATPase activity. The peroxynitrite-induced actomyosin impairment correlated with structural modifications that decrease the thermal stability of both actin and myosin leading to partially unfolded states. The results suggest a major role for the highly reactive cysteines on actin and on myosin and also for some critical methionines on G-actin. 3-nitrotyrosine does not contribute significantly to the observed functional alterations.

Introduction

Peroxynitrite (PN) is increasingly recognized as playing a major role in the skeletal and cardiac muscle dysfunction. This is supported by detection of protein 3-nitrotyrosine (3-NT), a protein modification produced by the reaction of PN with tyrosine, on skeletal and cardiac muscle proteins during aging [1] or in diseases associated with myocardial inflammation or ischemia/reperfusion insults [2,3]. Although these studies point to a correlation of protein nitration with functional and structural modifications, the mechanism by which PN may impair muscle contractility remains far from being elucidated. During inflammation or ischemia/reperfusion episodes, tissue cells are exposed to fluxes of PN ranging from submicromolar to micromolar concentrations [4]. Therefore, in order to mimic a chronic exposure to PN we have used 3-

morpholinosydnonimine (SIN-1), a compound that slowly decomposes in nitric oxide ($^{\cdot}NO$) and superoxide anion ($^{\cdot}O_2^-$), which react each other to produce $ONOO^-$. In this work, we report the functional and structural impairment of the myofibril proteins – myosin and actin - under the oxidative stress mediated by PN. In addition, the relevance of different protein modifications commonly produced by PN to the actomyosin impairment is evaluated.

Materials and Methods

Myosin and actin were isolated from rabbit skeletal muscle and the F-actin stimulated myosin ATPase activity measured spectrophotometrically using the coupled enzyme pyruvate kinase/lactate dehydrogenase assay as in previous works [5,6]. Differential Scanning Calorimetry measurements were performed on a Micro-Cal MC-2 operated at a scanning rate of 60ºC/h and under a nitrogen pressure of 1.5-2 kg/cm^2 during the scan. Titration of cysteines was done with DTNB, as previously indicated [5,6]. Methionine oxidation was assessed by SDS-PAGE densitometric analysis of the actin digestion products produced by a-chymotrypsin and subtilisin as in Dalle-Donne et al. [7]. The extent of 3-NT formation was assessed either spectrophotometrically or by Western-blotting as in Tiago et al. [5,6].

Results and Discussion

In vitro exposure of actin to micromolar peroxynitrite fluxes promoted an imbalance of the G-actin (monomer)/F-actin (filament) equilibrium which can efficiently block the stimulation of the myosin ATPase activity by actin. Furthermore, incubation of purified myosin with SIN-1 also resulted in inhibition of the myosin ATPase activity stimulated by actin. The IC_{50} values, obtained from the nonlinear least square fit of the data shown in Figure 1, take place at molar ratios of 6.12±0.86 and 7.351.53 mol SIN-1/mol of myosin and actin, respectively, showing that both proteins are highly sensitive to PN oxidation. The loss of myofibril actomyosin ATPase activity parallels a decrease of the thermal stability of both proteins. This has been shown by the decrease of the unfolding enthalpy (DH) and critical temperature (Tc) of SIN-1 treated myosin and G-actin in the DSC experiments (Table 1). Moreover, the lack of significant decrease in the cooperative unit (DH_V)

Table 1. Effect of SIN-1 treatment on the thermal stability of myosin and actin

Sample	Tc (ºC)	ΔH (kcal/mol)
Myosin (control)	47.6 ± 0.1	245 ± 6
Myosin + SIN-1 (1:10)	47.4 ± 0.1	189 ± 9
Myosin + SIN-1 (1:10) + GSH	47.7 ± 0.1	210 ± 6
Actin (control)	66.2 ± 0.5	183 ± 17
Actin + SIN-1 (1:10)	62.5 ± 0.5	116 ± 11
Actin + SIN-1 (1:10) + GSH	67.1 ± 0.5	149 ± 9

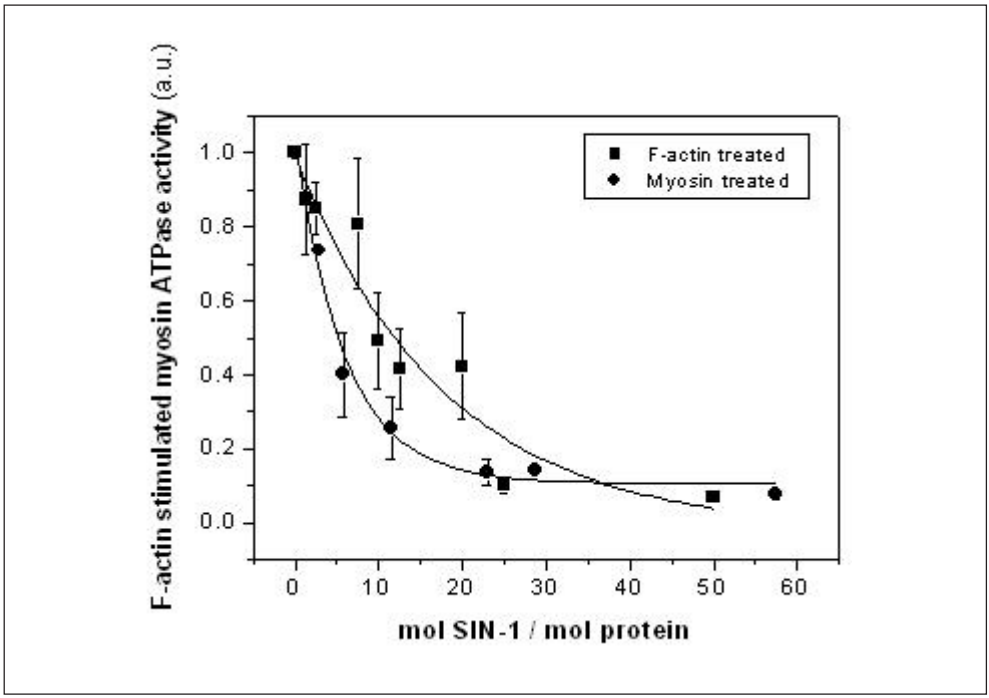

Figure 1. Inhibition of the myosin ATPase activity stimulated by actin upon SIN-1 treatment. Activity assays were done with F-actin (squares) or myosin subfragment-1 (circles) treated for 2h with different SIN-1 concentrations in 10mM Tris (pH 8.5), 25 mM KCl as indicated in Materials and Methods.

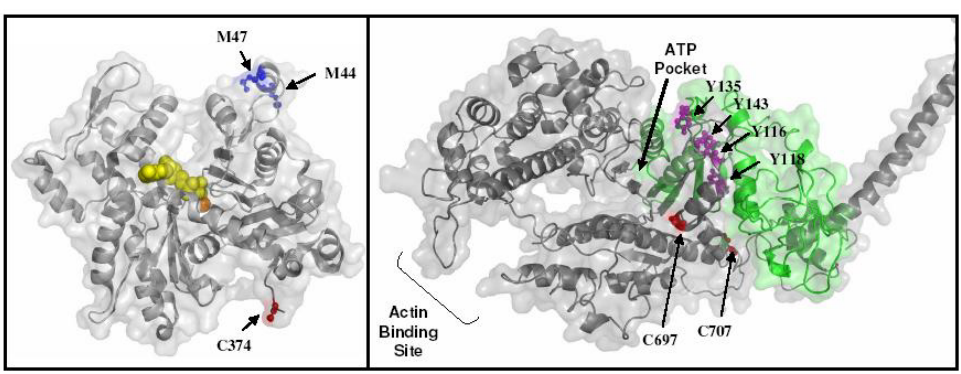

Figure 2. Molecular model of G-actin (PDB Id: 1J6Z) on the left, and myosin subfragment-1 (PDB Id: 2MYS) on the right, depicting the putative residues modified by PN. ATP and Ca^{2+} represented as yellow and orange spheres, respectively, are shown just to indicate the active site on the actin structure. Cyst and Met residues are represented in red and blue stick-and-balls, respectively. The 25kDa tryptic fragment of myosin is coloured green where it is indicated in purple the putative nitrated Tyr residues.

points out that PN is inducing partial unfolded states of myosin and G-actin which can be fully prevented by incubating the sample with GSH during SIN-1 treatment. To address the oxidative modifications accounting for the structural and functional impairment of the actomyosin complex under the oxidative stress induced by PN, different protein modifications commonly produced by PN were assessed as described in Materials and Methods. The results suggested a major role for Cys-707 and Cys-697 on myosin, two highly reactive cysteines located close to the nucleotide binding site (Figure 2) whose modification has been previously shown to have dramatic effects on the functional properties of myosin. 3-NT formation could be detected in the 25 kDa tryptic myosin fragment (Figure 2), consistent with the location of the four nitrated tyrosines in the myosin heavy chain extracted from cardiac muscle of aged rats [8]. However, the extent of this chemical modification is very low, i.e. less than 0.05 moles 3-NT/mole of myosin when 50% of the activity is already lost. On actin, besides the highly reactive Cys-374, we found a significant contribution of Met-44 and Met-47 to the observed impairment. While Cys-374 is located at the C-terminal region which is the binding site for several actin-binding proteins (including myosin), Met-44 and Met-47 are located in a loop implicated in monomer/monomer contact (Figure 2). Therefore, the modification of these critical residues by PN can directly affect the functional interaction with myosin as well as the G-actin/F-actin equilibrium. 3-NT formation do not seem to significantly contribute to the actomyosin ATPase activity impairment.

Acknowledgements

T. Tiago is supported by a post-doctoral grant (SFRH/BPD/20777/2004) from the Portuguese Foundation for Science and Technology (FCT). Work funded by Grant 3PR05A078 of Junta de Extremadura.

References

[1] J. KANSKI et al. Proteomic analysis of protein nitration in aging skeletal muscle and identification of nitrotyrosine-containing sequences in vivo by nanoelectrospray ionization tandem mass spectrometry, J Biol Chem, 280, 24261-24266 (2005).

[2] N.W. KOOY et al. Extensive tyrosine nitration in human myocardial inflammation: evidence for the presence of peroxynitrite, Crit Care Med, 25, 812-819 (1997).

[3] M.J. MIHM et al. Impaired myofibrillar energetics and oxidative injury during human atrial fibrillation, Circulation, 104, 174-180 (2001).

[4] M.P. MURPHY et al. Peroxynitrite: a biologically significant oxidant, Gen Pharmacol, 31, 179-186 (1998).

[5] T. TIAGO et al. Inhibition of skeletal muscle S1-myosin ATPase by peroxynitrite, Biochemistry, 45, 3794-3804 (2006).

[6] T. TIAGO et al. Peroxynitrite induces F-actin depolymerization and blockade of myosin ATPase stimulation, Biochem Biophys Res Commun 342, 44-49 (2006).

[7] I. DALLE-DONNE et al. The tert-butyl hydroperoxide-induced oxidation of actin Cys-374 is coupled with structural changes in distant regions of the protein, Biochemistry, 38, 12471-12480 (1999).

[8] S.J. HONG et al. Proteomic analysis of age dependent nitration of rat cardiac proteins by solution isoelectric focusing coupled to nanoHPLC tandem mass spectrometry, Exp Gerontol, 42, 639-651 (2007).