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## Susceptibility of Isolated Myofibrils to In Vitro Glutathionylation: Potential Relevance to Muscle Functions

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**In this study we investigated the molecular mechanism of glutathionylation on isolated human cardiac myofibrils using several pro-glutathionylating agents. Total glutathionylated proteins appeared significantly enhanced with all the pro-oxidants used. The increase was completely reversed by the addition of a reducing agent, demonstrating that glutathione binding occurs by a disulfide and that the process is reversible. A sensitive target of glutathionylation was  $\alpha$ -actin, showing a different reactivity to the several pro-glutathionylating agents by ELISA. Noteworthy, myosin although highly sensitive to the in vitro glutathionylation does not represent the primary glutathionylation target in isolated myofibrils. Light scattering measurements of the glutathionylated  $\alpha$ -actin showed a slower polymerisation compared to the non-glutathionylated protein and force development was depressed after glutathionylation, when the myofibrils were mounted in a force recording apparatus. Interestingly, confocal laser scanning microscopy of cardiac cryosections indicated, for the first time, the constitutive glutathionylation of  $\alpha$ -cardiac actin in human heart. Due to the critical location of  $\alpha$ -actin in the contractile machinery and to its susceptibility to the oxidative modifications, glutathionylation may represent a mechanism for modulating sarcomere assembly and muscle functionality under patho-physiological conditions in vivo.** © 2009 Wiley-Liss, Inc.

**Key Words:** glutathione, glutathionylated proteins, oxidative stress, actin, myofibrils

### Introduction

Protein glutathionylation is a dynamic process that is currently considered a mechanism of redox-mediated signal transduction, as well as a way for cells to store glutathione during oxidative stress and/or to protect critical protein cysteines from the irreversible oxidation, thus preventing permanent loss of function as a consequence of oxidative insult [Klatt and Lamas, 2000]. During oxidative stress, glutathione (GSH) is oxidized to GSSG either enzymatically (e.g. Glutathione peroxidase) or nonenzymatically. As endogenous levels of GSSG increase, cysteines moieties of target proteins can be glutathionylated by thiol disulfide exchange. Alternatively, protein thiols can be directly oxidized to form thyl radical or sulfenic acid, which can subsequently react with GSH to form the mixed disulfide (GS-Pro). To date, numerous proteins have been shown to be regulated by protein glutathionylation [Dalle-Donne et al., 2007]. A number have also been observed under basal conditions, suggesting their involvement in physiological signalling besides redox function regulation [Fratelli et al., 2002; Lind et al., 2002].

Reactive oxygen species (ROS) may be an important regulatory mechanism for normal contractile muscle apparatus [Andrade et al., 1998; Lamb and Posterino, 2003]. They are also implicated in many diseases, and play an important role in mediating cardiac hypertrophy [Amin et al., 2001], and during ischemia-reperfusion [Berlett and Stadtman, 1997; Bolli et al., 1988; Eaton et al., 2002; Canton et al., 2004].

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Some redox-sensitive thiols may represent putative “sensors” in the contractile filaments by activating redox-sensitive signalling pathways [Andrade et al. 1998; Lamb and Posterino 2003]. However, the identity of these “redox sensors” is not yet determined, although critical reactive cysteines are found in many key cardiac proteins including those implicated in ATP production, ionic homeostasis, signal transduction, transcription and excitation-contraction coupling [Choi et al., 2001; Eu et al., 2000; Shattock and Matsuura 1993].

Contractile function is sensitive to changes in cellular redox balance and the treatment of intact and permeabilized skeletal muscle fibers with various oxidants results in changes in force and  $\text{Ca}^{++}$  sensitivity [Andrade et al., 1998; Mihm et al., 2001; Lamb and Posterino, 2003; Posterino et al., 2003; Canton et al., 2004]. Until now, the molecular basis behind the observed changes remained unknown, but glutathione could take part in these oxidative effects [Lamb and Posterino, 2003; Murphy et al., 2008] and protein glutathionylation may represent a mechanism by which glutathione can modulate sarcomere functions depending on the redox state of the tissue.

In this study, we investigated the effect of glutathionylation on isolated muscle myofibrils. The aim was threefold: to analyse the susceptibility to the oxidation of the isolated myofibrils using various pro-glutathionylating agents, to identify the potential targets of glutathionylation, and to evaluate the functional effects of myofibrillar glutathionylation.

## Materials and Methods

### Chemicals

Rat anti-GSPro, secondary goat anti-rat conjugated with fluorescein isothiocyanate (FITC), goat anti-mouse conjugated with tetramethylrhodamine isothiocyanate (TRITC) were obtained from Chemicon International Inc. (Temecula, CA); mouse anti-cardiac actin antibody was from Research Diagnostics (Concord, MA). Mouse anti-slow myosin antibody was from Novocastra (Newcastle, UK). BCA-protein assay was from Sigma-Aldrich (St. Louis, MI).

Rabbit skeletal muscle  $\alpha$ -actin and bovine cardiac  $\alpha$ -actin were from Cytoskeleton Inc. (Denver, CO). Human heart samples, obtained at autopsy 2 to 10 h after death, with the consent of the Institutional Ethical Committee, were frozen in liquid nitrogen for biochemical study and in liquid nitrogen-cooled isopentane for immunofluorescence analysis.

### Myofibrils Preparation for Biochemical Studies

In total, 200 mg of cardiac tissue were homogenized in cold imidazole buffer (0.3 M sucrose, 10 mM imidazole, pH 7) and centrifuged for 15 min at  $17,000 \times g$  at  $4^{\circ}\text{C}$ , as reported by Cappelli et al. [1988].

### Determination of Total Protein Glutathionylation by HPLC

Totally, 75  $\mu\text{g}/\text{ml}$  cardiac myofibrils were incubated for 2 h at  $37^{\circ}\text{C}$  with 1 mM GSSG, 1 mM GSH + 1mM diamide, and 1 mM GSH + 1 mM  $\text{H}_2\text{O}_2$ . To 50  $\mu\text{l}$  glutathionylated myofibrils were added 25  $\mu\text{l}$  of 12% sulfosalicylic acid; protein pellets were then dissolved in 75  $\mu\text{l}$  of 0.1 N NaOH and protein bound glutathione (GS-Pro) determined. The derivatization and chromatography procedures were performed, as previously reported [Pastore et al., 2003].

### Analysis of Glutathione-Conjugates by Western Blot

Totally, 20  $\mu\text{g}$  of cardiac myofibrils were incubated with 1 mM GSSG, 1 mM GSH + 1 mM diamide, 1 mM GSH + 1 mM  $\text{H}_2\text{O}_2$  for 15 min at  $37^{\circ}\text{C}$ . Aliquots of glutathionylated samples were subsequently treated with 5 mM DTT. All samples were applied onto 8% non-reducing SDS-polyacrylamide gel electrophoresis and the proteins transferred onto a nitrocellulose membrane overnight at 70 mA. The membrane was blocked with 5% non-fat dry milk in TBST (100 mM NaCl and 10 mM Tris-HCl, pH 7.8, containing 0.1% Tween 20) for 2 hours, at room temperature, and probed with anti-GSPro antibody (1/1000), anti-actin antibody (1/1000), anti-myosin antibody (1/4000).

### Detection of $\alpha$ -Cardiac Actin, $\alpha$ -Skeletal Actin and Myosin Glutathionylation by ELISA

96-well Maxi-Sorp ELISA plates (Nunc Inc., Rochester, NY) were coated with 1  $\mu\text{g}/\text{ml}$   $\alpha$ -cardiac or  $\alpha$ -skeletal actin, or myosin in pH 9.6 carbonate buffer for 30 min at  $22^{\circ}\text{C}$  as reported by Johansson and Lundberg [2007]. GSSG or GSH were incubated with diamide or  $\text{H}_2\text{O}_2$  as indicated and the samples incubated 15 min at  $22^{\circ}\text{C}$ . Washing the wells four times with PBST terminated all reactions. Actin and myosin glutathionylation was detected with a monoclonal anti-protein bound glutathione antibody. The antibody was diluted 1:1000 in PBST supplemented with 1% (w/v) fraction V BSA and added to the samples for 1 h incubation. The plates were washed four times in PBST and incubated for 1 h with an anti-mouse biotin-conjugated antibody diluted 1:1000 in PBST, containing 1% (w/v) fraction V BSA. The plates were washed four times in PBST, and streptavidin (1:1000) was added for 30 min and revealed with Sigma Fast OPD (St. Louis, MI). Absorbance at 450 nm was determined using a micro-titre plate reader (Wallac, MA). No signal was detected in the wells that had not been coated with actin, independently from the combinations with the oxidants. Accordingly, these reagents do not bind to the plates and interfere with the assay.

### Light Scattering of $\alpha$ -Cardiac Actin

The polymerisation of actin was monitored by following the change in light scattering after incubation of the protein with 1 mM GSSG, 1 mM GSH + 1 mM diamide, and 1

mM GSH + 1 mM H<sub>2</sub>O<sub>2</sub>. Light scattering measurements were performed on a Horiba (Kyoto, Japan) LB - 550 dynamic light scattering nanoparticle size analyser, equipped with a 650 nm, 5 mW laser diode. All experiments were carried out at 25°C by thermostating the sample holder with an external water-circulating bath. Data analysis was performed using the accompanying software based on a Fourier-transform deconvolution procedure.

### Myofibril Preparation and Force Recording Apparatus

Single myofibrils or bundles of two to three myofibrils were prepared from skeletal muscle by homogenisation of glycerinated rabbit psoas muscles, as described previously [Tesi et al., 2002a,b]. Briefly, a small volume of the myofibril suspension was transferred to a temperature-controlled chamber (15°C) filled with relaxing solution (pCa 8.0) and mounted on an inverted microscope. Selected preparations (single myofibrils or bundles of few myofibrils, 25–80 µm long, 1–2 µm wide) were mounted horizontally between two glass microtools. One tool was connected to a length-control motor that could produce rapid (<1 ms) length changes. The second tool was a calibrated cantilevered force probe (2–6 nm/nN; frequency response 2–5 kHz). Force was measured from the deflection of the image of the force probe projected on a split photodiode. Average sarcomere length and myofibril diameter were measured from video images (ca 1800×). The initial sarcomere length of the preparations was set around 2.5 µm. Myofibrils were activated and relaxed by rapid translation of two continuous streams of relaxing and activating solutions of different pCa flowing by gravity from a stepper motor controlled double-barrelled glass pipette placed within 0.5–1 mm of the preparation. The solution change took place with a time constant of 2–3 ms and was complete in less than 5 ms. A release-restretch protocol was used to measure the rate of force redevelopment (kTR) at saturating [Ca<sup>2+</sup>] [Brenner, 1988]. Experiments were performed at 15°C. Activating and relaxing solutions, calculated as previously described [Tesi et al., 2002a,b], were at pH 7.00 and contained 10 mM total EGTA (CaEGTA/EGTA ratio set to obtain pCa 8.0 and 4.5), 5 mM MgATP, 1 mM free Mg<sup>2+</sup>, 10 mM Mops, propionate and sulphate to adjust the final solution to an ionic strength of 200 mM and monovalent cation concentration of 155 mM. Creatine phosphate (CP; 10mM) and creatine kinase (200 units ml<sup>-1</sup>) were added to all solutions. Contaminant inorganic phosphate (Pi) from spontaneous breakdown of MgATP and CP was reduced to less than 5 µM by a Pi scavenging system (purine-nucleoside-phosphorylase with substrate 7-methylguanosine) [Tesi et al., 2002a,b]. All solutions to which the samples and myofibrils were exposed contained a cocktail of protease inhibitors including (10 µM) leupeptin, (5 µM) pepstatin, (200 µM) PMSF and (10 µM) E64, (500 µM) NaN<sub>3</sub>. When indicated, 1 mM GSH+ 1 mM diamide was added to activating solution (pH 7.0).

Myofibrils were subjected to several activation/relaxation cycles, in control conditions or in the presence of mM GSH+ 1 mM diamide in activating solution. Maximal activated force (P<sub>0</sub>), rate of force development (*k*<sub>ACTIV</sub>) and redevelopment (kTR) were measured and compared in usually at least three successive cycles from the same myofibril. As previously reported [Tesi et al., 2002a,b], in rabbit psoas myofibrils (15°C, control conditions), rundown of force following four to five activation/relaxation cycles was less than 10%.

### Immunofluorescence and TPE Confocal Microscopy Analyses

Transversal cardiac muscle cryosections (5 µm) from human hearts (*n* = 2) were cold acetone fixed (–20°C), blocked with 5% normal goat serum in PBS/BSA1% for 30 min, and labelled with polyclonal rat anti-GSPro antibody (1/25). After washing in PBS, slides were incubated with secondary antibody goat anti-rat FITC conjugated. Double staining was performed using mouse anti-cardiac actin (1/10) and revealed with goat anti-mouse TRITC conjugated. Negative controls were performed using PBS/BSA without the primary antibody. Image acquisition was performed using a two-photon excitation (TPE) microscope built around a conventional confocal laser-scanning microscope Nikon-C1-Plus (Nikon Instruments, Florence, Italy). TPE fluorescence of the dyes was detected using a 750 nm Ti: sapphire ultrafast laser source (Mai Tai Laser 750-850, Spectra Physics, CA) at approximately 6 mW of average power in the focal plane. The fluorescence signal, collected by the same objective and selected by a filter (HQ535-50, Chroma Inc., Brattleboro, VT), is fed to a multimode fiber that brings the light to a photomultiplier (R928, Hamamatsu, Milan, Italy) in the C1 plus controller. The images of samples were acquired at (1024 × 1024 pixels) with residence times in the range of 9.6 ms per pixel takes 0.24 s. and were analysed with EZC1 software (Nikon Instruments, Florence, Italy). The software with the algorithm and the numerical analysis with “Image J” version 1.33u (N.I.H., USA) were used to calculate the co-localization.

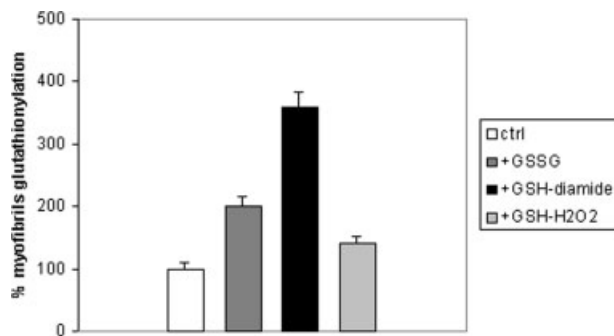
### Statistical Analysis

Data are expressed as mean ± SD. The comparison between values obtained in treated samples and controls was performed by the Student's *t*-test for unpaired data.

## Results

### Glutathionylation of Cardiac Myofibrils In Vitro

Although the relevance of ROS action on the acto-myosin system remains to be elucidated, oxidative stress has been found to cause multiple changes in myocyte structure and function, leading to hypertrophy and failing heart [Keith et al., 1988; Sawyer et al., 2002]. Furthermore, exposure of rat skeletal muscle fibres to H<sub>2</sub>O<sub>2</sub> had profound effects on



**Fig. 1. Effect of various pro-oxidants (GSSG, GSH+diamide, GSH+ H<sub>2</sub>O<sub>2</sub>) on protein glutathionylation of isolated human cardiac myofibrils.** GSH bound to the proteins (GS-Pro) of isolated cardiac myofibrils was determined by HPLC analysis after incubating 75 µg/ml myofibrils for 2 h at 37°C with 1 mM GSSG, 1 mM GSH + 1 mM diamide, and 1 mM GSH + 1 mM H<sub>2</sub>O<sub>2</sub>. All data are represented as the mean ± SD of three experiments. \**P* < 0.05 vs. wild type. For details, see “Materials and Methods”.

contractile function, interestingly depending on the presence of the reduced form of GSH [Lamb and Posterino, 2003].

In this study we analysed the effect of various pro-oxidants (GSSG, GSH+diamide, GSH+ H<sub>2</sub>O<sub>2</sub>) on protein glutathionylation of isolated human cardiac myofibrils. As reported in Fig. 1, total glutathionylated protein level was significantly enhanced in cardiac myofibrils, ranging from  $1.2 \pm 0.1$  to  $2.4 \pm 0.36$ ,  $4.3 \pm 1.08$ ,  $1.7 \pm 0.17$  (mean ± SD) nmol/mg prot., after incubation with 1 mM GSSG, 1 mM GSH + 1 mM diamide, 1 mM GSH + 1 mM H<sub>2</sub>O<sub>2</sub>, respectively. These increases were completely reversed by the subsequent addition of 1 mM DTT (data not shown).

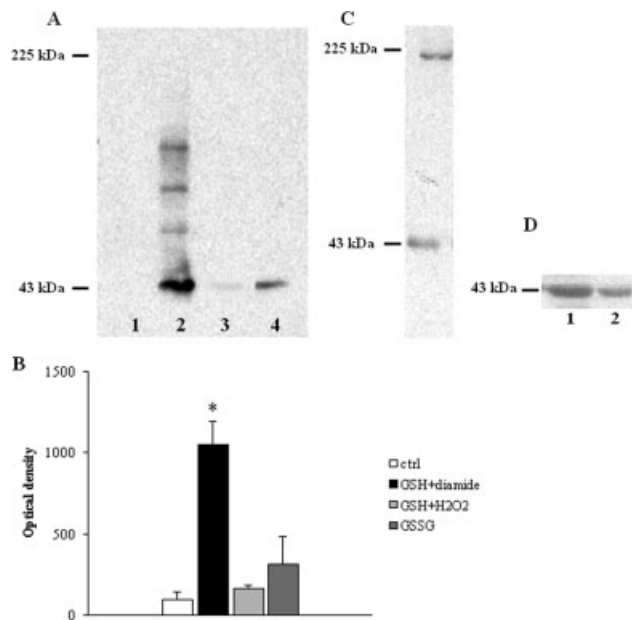
To attempt to identify the proteins mostly glutathionylated in isolated myofibrils, we performed Western blot analysis at non-reducing conditions (Fig. 2A) and we confirmed the different myofibrillar proteins’ sensitivity to the various oxidants, especially after oxidation by GSH+diamide (lane 2). Noteworthy, the human skeletal myofibrils show exactly the same electrophoretic pattern of protein glutathionylation than that obtained with the isolated cardiac myofibrils (data not shown).

As the majority of proteins constituting the myofibrils are actin (about 20%) and myosin (43%) (37) and given that the electrophoresis mobility of the most glutathionylated protein was comparable to that of the pure cardiac actin (Fig. 2C), we immunoprecipitated the human cardiac myofibrils with anti-GSPro antibody and probed by an anti-cardiac isoform of α-actin antibody. Interestingly, α-cardiac actin appears glutathionylated in human heart even under physiological conditions (Fig. 2D lane 1), whereas myosin, which is highly reactive to the in vitro glutathionylation [Passarelli et al., 2008], loses its reactivity when organized in a sarcomeric structure. These findings support the results recently obtained by Prochniewicz et al. [2008] showing that H<sub>2</sub>O<sub>2</sub> treatment on rabbit psoas muscle does not affect myosin cysteines, though it causes a reversible decrease in fiber contractility. Thus, as for the cytoskeletal β-actin [Fratelli

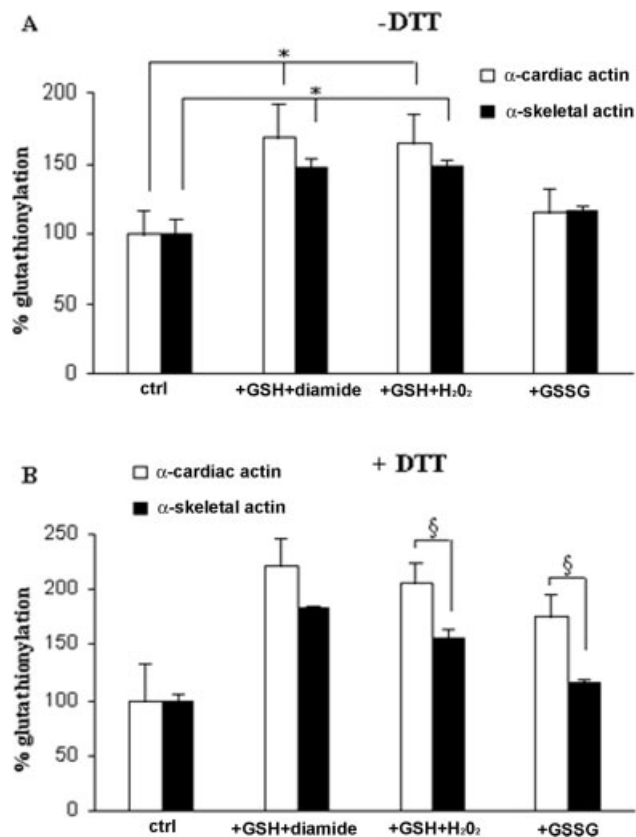
et al., 2002; Dalle-Donne et al., 2003; Fiaschi et al., 2006], even α-actin may constitute a direct target for oxidative modification in human heart either in physiological and pathological conditions.

### Different Reactivity to Glutathionylation of α-Cardiac and α-Skeletal Muscle Actins

In order to evaluate the oxidant susceptibility of the α cardiac and α skeletal muscle isoforms of actin, we tested some pure commercial actins with several pro-oxidants using an enzyme-linked immunosorbant assay previously described by Johansson and Lundberg [2007]. We found that both the α-actin isoforms are sensitive to the various oxidants (Fig. 3). In particular, they appear significantly sensitive to the in vitro glutathionylation by GSH combined with diamide and H<sub>2</sub>O<sub>2</sub>, but both appear less glutathionylable by GSSG (A). As expected, the ELISA signal for glutathionylation was decreased to background levels when DTT was added after glutathionylation (data not shown), thus indicating that glutathione is bound to actin by a disulfide, and that the process is reversible. The different reactivity with the pro-glutathionylating agents is maintained even when the



**Fig. 2. Identification of α-cardiac actin in isolated myofibrils.** (A) 20 µg of cardiac myofibrils without treatment (lane 1) and after incubation for 15 min at 37°C with 1 mM GSH + 1 mM diamide (lane 2), 1 mM GSH + 1 mM H<sub>2</sub>O<sub>2</sub> (lane 3) and 1 mM GSSG (lane 4) were separated by not-reducing SDS-PAGE and transferred to nitrocellulose. The glutathione conjugates were probed with a monoclonal anti-GSPro antibody (1/1000). (B) Densitometry analysis of the bands corresponding to cardiac actin. (C) Western blot analysis of isolated cardiac myofibrils revealed by anti-cardiac actin antibody (1/1000) and re-incubated with anti-myosin antibody (1/4000). (D) Immunoblot detection with anti-GSPro antibody of immunoprecipitated cardiac actin from isolated human myofibrils (lane 1; lane 2, pure cardiac actin). The blots are representative of one of three independent experiments. For details, see “Materials and Methods”.



**Fig. 3. Different reactivity to glutathionylation of  $\alpha$ -cardiac and  $\alpha$ -skeletal muscle actins by ELISA analysis.** (A) 1  $\mu$ g/ml  $\alpha$ -cardiac and  $\alpha$ -skeletal actin were glutathionylated by 1 mM GSH + 1 mM diamide, 1 mM GSH + 1 mM H<sub>2</sub>O<sub>2</sub> and 1 mM GSSG for 15 min at 22°C, and detected using an anti-GSPro ELISA. \**P* < 0.05 vs. wild type. (B)  $\alpha$  cardiac and  $\alpha$  skeletal actin were pre-incubated with 1 mM DTT and then glutathionylated by 1 mM GSH + 1 mM diamide, 1 mM GSH + 1 mM H<sub>2</sub>O<sub>2</sub> and 1 mM GSSG, as reported in (A). §*P* < 0.05 vs.  $\alpha$ -skeletal actin. All data are represented as the mean  $\pm$  SD of three experiments. For details, see “Materials and Methods”.

$\alpha$ -muscle actins are previously treated with DTT, to reduce any disulfides, and then incubated with the oxidants (Fig. 3B). But, differently from the skeletal actin, the cardiac isoform appears more reactive with GSSG when pre-reduced with DTT (Fig. 3B). Therefore, as for  $\beta$ -actin [Klatt and Lamas 2000], also for the human  $\alpha$ -actin we can hypothesize the formation of a cysteinyl sulfenic acid intermediary, which reacts with GSH after oxidation (diamide, H<sub>2</sub>O<sub>2</sub>) leading to protein glutathionylation. In the presence of a reducing agent (DTT) also GSSG becomes a good glutathionylating agent for  $\alpha$ -cardiac actin, thus explaining how glutathionylation can occur even in a reducing intracellular environment, where the majority of protein thiols are reduced.

In addition, our study shows that myosin does not constitute the primary glutathionylation target in cardiac human myofibrils (Fig. 2), although the pure protein is in vitro highly sensitive to glutathionylation, as we demonstrated in a recent paper [Passarelli et al., 2008] and confirmed in this study by ELISA analysis (Fig. 4). Like cardiac actin, even

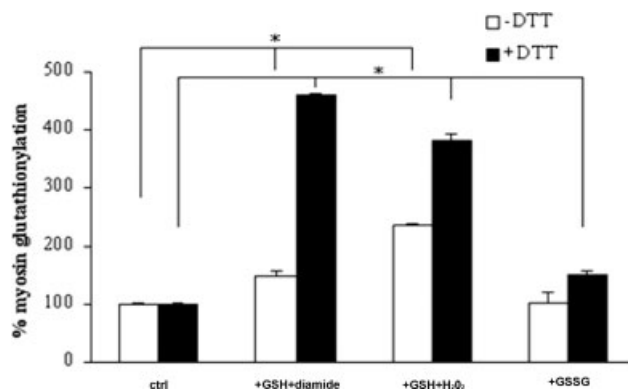
myosin is more glutathionylable when previously DTT-treated.

### Effect of Glutathionylation on Actin Polymerisation

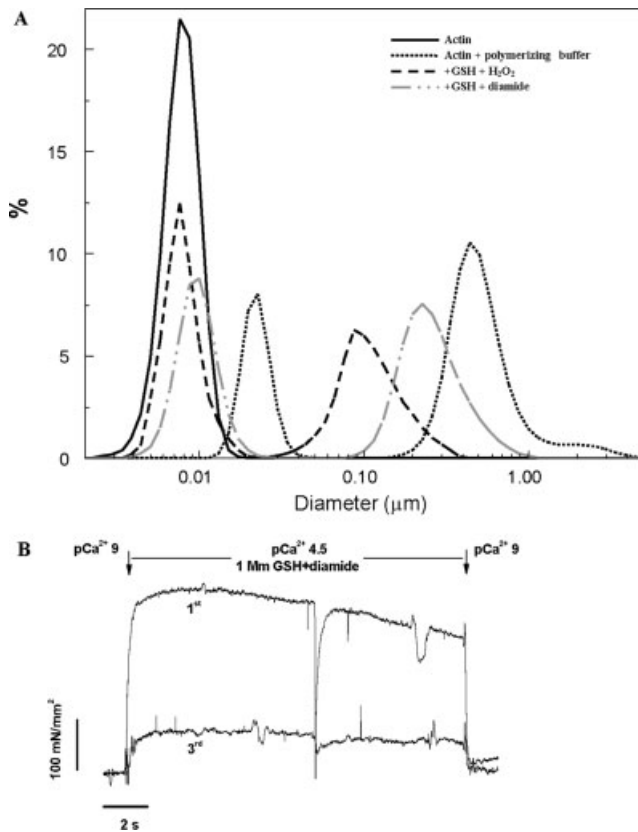
ROS take part in the control of actin microfilament dynamics causing rapid and dramatic changes in cell morphology and motile activity [Saurin et al., 2004; Poole et al., 2004; Stone and Yang, 2006]. In particular, H<sub>2</sub>O<sub>2</sub> regulates  $\beta$ -actin polymerisation in A431 cells by modulating its glutathionylation [Wang et al., 2001]. Therefore, in light of our in vitro data showing the considerable reactivity of  $\alpha$  actin with the oxidants, we examined the effect of glutathionylation on purified  $\alpha$ -cardiac actin polymerisation by light scattering (Fig. 5A). The results show that the particles size of individual actin subunits (solid line, Table I) is consistent with those reported in literature (~7 nm in diameter for globular actin) and that the incubation for 15 min of  $\alpha$  cardiac actin with GSH + H<sub>2</sub>O<sub>2</sub> (black dashed line) and GSH + diamide or GSSG (grey dashed line) resulted in an actin which polymerised slower than the non-glutathionylated protein (dotted line). In particular, in the presence of GSH + diamide, GSH + H<sub>2</sub>O<sub>2</sub> and GSSG about 50% of the protein molecules still retain the native dimensions. Therefore, the glutathionylation of cardiac  $\alpha$  actin may be considered a regulatory mechanism of its polymerisation.

### Effect of Glutathionylation on Isolated Myofibrils Force Development

The effect of glutathionylation on the contraction of isolated myofibrils of skeletal muscle was studied in force recording apparatus (Fig. 5B), by comparing maximal force level and the kinetics of force development of three successive activation-contraction cycles in presence of 1 mM GSH + 1 mM diamide in the activating solution. As shown in Fig. 5B and



**Fig. 4. Detection of myosin glutathionylation by ELISA analysis.** A total of 1  $\mu$ g/ml myosin were glutathionylated by 1 mM GSH + 1 mM diamide, 1 mM GSH + 1 mM H<sub>2</sub>O<sub>2</sub> and 1 mM GSSG for 15 min at 22°C with or without pre-treating by 1 mM DTT and myosin glutathionylation detected by ELISA using the anti-GSPro antibody. All data are represented as the mean  $\pm$  SD of three experiments. \**P* < 0.05 vs. wild type. For details, see “Materials and Methods”.



**Fig. 5. (A) Effect of glutathionylation on actin polymerisation.** Totally, 200 µg of  $\alpha$ -cardiac actin were incubated for 15 min with polymerizing buffer (dotted line) or with 1 mM GSH + 1 mM  $H_2O_2$  (dashed line), 1 mM GSSG and 1 mM GSH + 1 mM diamide (grey dashed line), and the change in light scattering was monitored at 25°C with respect to the native protein profile (solid line), as reported in “Material and Methods”. The profiles are representative of one of three independent experiments. **(B) Effect of glutathionylation on isolated myofibrils force development.** Force generation and relaxation was measured in skeletal myofibrils treated with 1 mM GSH + 1 mM diamide, as reported in “Materials and Methods”. Representative force responses of a rabbit psoas myofibril repetitively maximally activated and fully relaxed by fast solution switching (pCa changes at arrows as indicated) in the presence of 1 mM GSH + 1 mM diamide (Temperature 15°C). Maximal force progressively decreased and reached a quasi-steady state after three activation-relaxation cycles. Time interval between activations about 30 s. Initial sarcomere length 2.53 µm. For details, see “Materials and Methods”.

Table II, after about 1 min total exposure to GSH/diamide, force development and its kinetics were strongly depressed. Initial force in the first contraction in the presence of 1 mM GSH + 1 mM diamide, as well as the kinetics of force development ( $K_{activ}$ ) and redevelopment ( $k_{TR}$ ), were very close to control values, as measured in present experiments or from previous studies in similar conditions [Tesi et al., 2002a,b]. The inhibition of force development was strongly time dependent and approached 100% after longer exposures: batches of myofibrils exposed for about 20 min to 1 mM GSH + 1 mM diamide showed no force development. The effect was fully reversible after centrifuge washing in the

presence of 1 mM DTT (see Table II), and the run down of force following three repetitive activations was absent, as previously observed in control rabbit psoas myofibrils in similar conditions [Tesi et al., 2002a,b]. Therefore, due to the critical location of  $\alpha$ -actin, its glutathionylation may represent a mechanism for regulating sarcomere assembly and muscle functionality in vivo.

### Glutathionylation of Cardiac Myofibrils In Vivo

Under physiological conditions, protein glutathionylation may represent an antioxidant device that reduces the impact of oxidative stress, but at the same time it can represent a redox-signal of protein regulation. Thus, we analysed in vivo actin glutathionylation examining cardiac human cryosections by TPE confocal microscopy. As reported in Fig. 6, the GSPro immunostaining showed a longitudinally oriented localization in cardiac myofibers (A), and significant overlapping areas with cardiac actin were detected in double labeling experiments (C, D). These findings confirm those obtained by immunoprecipitating the isolated cardiac myofibrils and highlight a physiological role for glutathionylation in cardiac tissue.

### Discussion

The progressive glutathionylation of key proteins is proposed as a molecular switch by which cells respond in an immediate and reversible fashion to oxidative stress. These redox-sensitive thiols may constitute putative “sensors” in the contractile apparatus for changes in intracellular ROS levels by activating redox-sensitive signalling pathways [Andrade et al., 1998; Lamb and Posterino, 2003]. Recently, Prochniewicz et al. [2008] reported that oxidation by 5 mM  $H_2O_2$  decreased fiber contractility (isometric force and shortening velocity) in rabbit psoas muscle without significant changes in the enzymatic activity of myofibrils and isolated myosin. The authors showed that  $H_2O_2$  treatment does not affect myosin cysteines, particularly the most reactive Cys 707. The functional tests here reported show for the first time a direct effect of glutathionylation on isolated skeletal myofibrils, with depression of force development occurring in a

**Table I. Experimental Particle Size Distribution Obtained by Light Scattering Measurements**

Sample	Fraction of molecules with diameter > 15 nm (%)	Center of particle size distribution/s (nm)
$\alpha$ -cardiac actin	5	7.6
+polymerizing buffer	98	22.0 445
+GSH + $H_2O_2$	47	7.4 87
+GSSG or + GSH + diamide	55	10.0 200

**Table II. Maximum Tension ( $P_0$ ) and Kinetic Parameters of Force Development ( $k_{\text{ACTIV}}$ ) and Redevelopment ( $k_{\text{TR}}$ ) in Rabbit Psoas Myofibrils in Control Conditions (First Contraction), in Presence of 1 mM GSH + 1 mM Diamide (First and Third Contraction) or After 10-min Exposure to 1 mM GSH + 1 mM Diamide Followed by Centrifuge Washing and Resuspension in Rigor Solution (First and Third Contraction)**

	$P_0$ mN·mm <sup>-2</sup>	$k_{\text{ACTIV}}$ s <sup>-1</sup>	$k_{\text{TR}}$ s <sup>-1</sup>
Control conditions			
1st contraction	583 ± 95 (5)	5.95 ± 0.55 (5)	7.37 ± 0.60 (5)
1 mM GSH + 1 mM diamide			
1st contraction	436 ± 56 (6)	6.38 ± 0.73 (5)	7.97 ± 0.57 (5)
3rd contraction	80 ± 60 (6)	3.23 ± 0.52 (4)	3.61 ± 1.16 (4)
$P_0$ , $k_{\text{ACTIV}}$ or $k_{\text{TR}}$ 3rd vs 1st contraction	0.19 ± 0.02 (4)	0.54 ± 0.15 (4)	0.46 ± 0.16 (4)
1 mM GSH + 1 mM DIAMIDE after washing and resuspension in rigor			
1st contraction	344 ± 126 (3)	6.18 ± 0.49 (3)	7.43 ± 0.83 (3)
3rd contraction	352 ± 118 (3)	7.41 ± 0.52 (3)	8.60 ± 0.20 (3)
$P_0$ , $k_{\text{ACTIV}}$ or $k_{\text{TR}}$ 3rd vs 1st contraction	1.05 ± 0.09 (3)	1.04 ± 0.13 (3)	0.94 ± 0.04 (3)

Results are expressed as Mean (±SE, n).

reversible manner and without affecting the sarcomere integrity of the preparation. Furthermore, our results show that, under condition of oxidative stress,  $\alpha$ -actin is particularly sensitive to bind glutathione in isolated cardiac and skeletal myofibrils, whereas myosin, which is highly reactive to the in vitro glutathionylation [Passarelli et al., 2008], seems to lose its reactivity to glutathionylation when organized in a sarcomeric structure.

Previous studies, performed in vitro and after post-ischemic reperfusion [Powell et al., 2001; Canton et al., 2004], have already indicated actin as a major target for oxidative modifications among the myofibrillar proteins, but the present study is the first to show its physiological glutathionylation in human heart and its sensitivity to GSH when combined with H<sub>2</sub>O<sub>2</sub> and diamide in isolated myofibrils. Moreover, our findings show that the glutathionylation of  $\alpha$  cardiac actin occurs non-enzymatically, but via spontaneous oxidation of a cysteinyl residue to a cysteinyl sulfenic acid intermediary, a mechanism previously reported by Johansson and Lundberg for the cytoskeletal  $\beta$ -actin [Johansson and Lundberg, 2007] and by Dalle-Donne et al. for rabbit skeletal muscle actin [Dalle-Donne et al., 2003b].

The high reactivity of  $\alpha$  cardiac actin with H<sub>2</sub>O<sub>2</sub> acquires particular relevance in vivo, where hydrogen peroxide has been proposed as the major redox signal for formation of cysteinyl sulfenic acids under physiological conditions [Saurin et al., 2004; Poole et al., 2004]. Indeed, a mild oxidation of the actin cysteine thiols by H<sub>2</sub>O<sub>2</sub> may activate the protein to be glutathionylated even at physiological levels of GSH. In addition, the high oxidative reactivity of the protein when pre-incubated with DTT enables us to explain how glutathionylation may occur also in a predominantly reducing cellular environment.

H<sub>2</sub>O<sub>2</sub> takes part in the control of actin microfilament dynamics causing rapid and dramatic changes in the morphol-

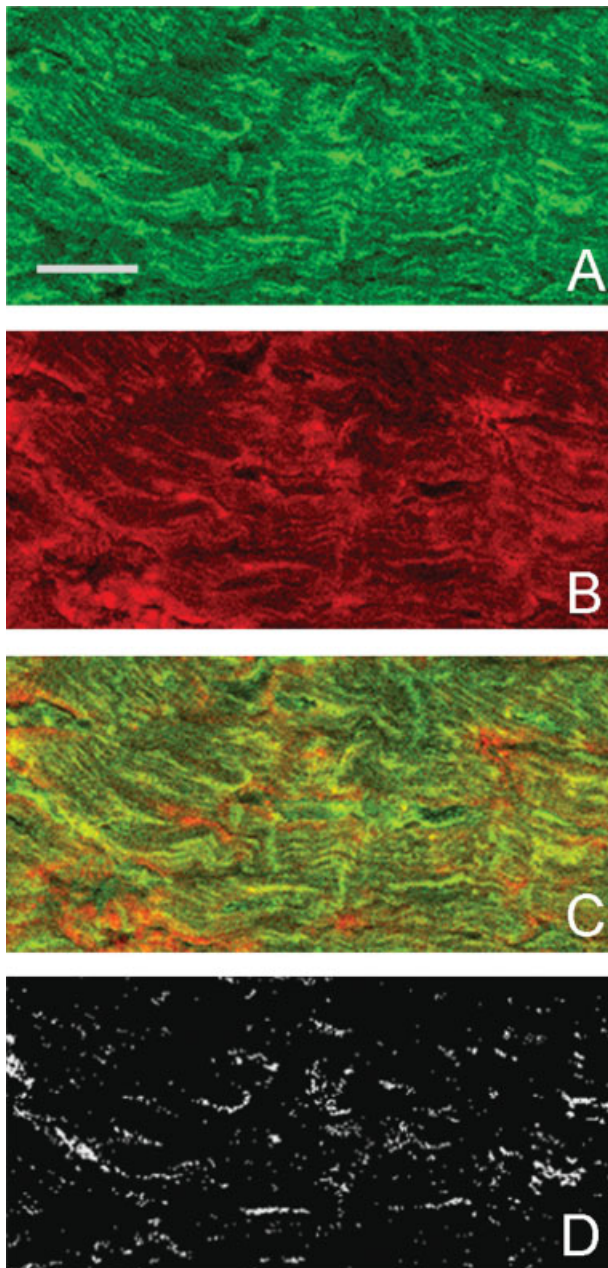
ogy and motile activity of eukaryotic cells [Saurin et al., 2004; Stone and Yang, 2006]. H<sub>2</sub>O<sub>2</sub> is also responsible of actin glutathionylation in human epidermal carcinoma A431 cells, where it regulates  $\beta$ -actin polymerisation [Wang et al., 2001]. By light scattering, we found that  $\alpha$ -cardiac actin polymerised slower than the native protein when in vitro glutathionylated. Thus, as for the cytoskeletal  $\beta$ -actin [Fratelli et al., 2002; Dalle-Donne et al., 2003; Fiaschi et al., 2006] and for the skeletal muscle actin [Dalle-Donne et al., 2003b], even  $\alpha$ -actin could constitute a direct target for oxidative modification in human heart and its glutathionylation may represent a mechanism by which glutathione can modulate sarcomere functions depending on the redox state of the tissue.

Actin was shown to be oxidatively modified in several pathophysiological states [Aksenov et al., 2001; Eaton et al., 2002; Canton et al., 2004], and glutathionylated actin was found in a rat model of ischemia-reperfusion [Chen and Ogut, 2006] and in fibroblasts of patients with a neurodegenerative disease [Pastore et al., 2003], where we reported an impairment of the cytoskeletal functions following  $\beta$ -actin glutathionylation. Axonal degeneration caused by the oxidation of actin has also been reported in a mouse model of amyotrophic lateral sclerosis [Collard et al., 1995], and high levels of oxidized actin were found in brain extracts of patients with Alzheimer's disease [Aksenov et al., 2001].

Therefore, given the particular sensitivity of  $\alpha$ -actin to bind glutathione in isolated cardiac and skeletal myofibrils, we propose actin as a redox-sensor of the sarcomere functions in human muscle and glutathione as a new protagonist in the patho-physiological signalling underlying muscle performance.

In conclusion, although until now the relevance of ROS action on the acto-myosin system remains to be elucidated, nevertheless our study may provide a basis for understanding





**Fig. 6. Glutathionylation of human cardiac myofibrils in vivo.** Representative images showing constitutive glutathionylation in 5- $\mu$ m cryosections of human hearts ( $n = 2$ ) by immunofluorescence confocal analysis. (A) GSPro labelling followed the longitudinal orientation of the myofibrillar network. The double staining (C) performed with an anti  $\alpha$ -actin antibody against cardiac actin (B) showed numerous areas of co-localization (D). Bar: 20  $\mu$ m.

the direct effect of glutathionylation on the myofibrillar structure and give important insights into the redox-mechanisms modulating muscle functions.

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