Articles in PresS. Am J Physiol Cell Physiol (December 14, 2016). doi:10.1152/ajpcell.00334.2016

C-00334-2016 Revised

S-Nitrosylation and S-Glutathionylation of Cys134 on troponin I have opposing competitive actions on Ca^{2+} sensitivity in rat fast-twitch muscle fibers

TL Dutka [1], JP Mollica [1], CR Lamboley [1,2], VC Weerakkody [3], DW Greening [3], GS Posterino [1], RM Murphy [3] & GD Lamb [1]

[1] School of Life Sciences, La Trobe University, Melbourne 3086, Victoria, Australia

[2] Institute of Sport, Exercise and Active Living (ISEAL), Victoria University, PO Box 14428, Melbourne, VIC 8001, Australia.

[3] Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne 3086, Victoria, Australia

Running Head: Opposing actions of nitrosylation and glutathionylation on TnI_f

Corresponding author:
Prof. Graham D. Lamb
School of Life Sciences, La Trobe University, Melbourne, Victoria, 3086, Australia
Telephone: +61-3-9479 2249
Facsimile: +61-3-9479 1551
E-mail: g.lamb@latrobe.edu.au

Keywords: muscle fatigue, skinned muscle fiber, contractile apparatus, oxidation

Word count: 7400

Abstract

Nitric oxide is generated in skeletal muscle with activity and decreases Ca^{2+}-sensitivity of the contractile apparatus, putatively by S-nitrosylation of an unidentified protein. We investigate the mechanistic basis of this effect and its relationship to the oxidation-induced *increase* in Ca^{2+}-sensitivity in mammalian fast-twitch (FT) fibers mediated by S-glutathionylation of Cys134 on fast troponin I (TnI_f). Force-[Ca^{2+}] characteristics of the contractile apparatus in mechanically-skinned fibers were assessed by direct activation with heavily Ca^{2+}-buffered solutions. Treatment with S-nitrosylating agents, S-nitrosoglutathione (GSNO) or S-nitroso-N-acetyl-penicillamine (SNAP), decreased pCa_{50} (=-$\log_{10}$ [Ca^{2+}] at half maximal activation) by ~-0.07 pCa units in rat and human FT fibers without affecting maximum force, but had no effect on rat and human slow-twitch fibers or toad or chicken FT fibers, which all lack Cys134. The Ca^{2+} sensitivity decrease was i) fully reversed with dithiothreitol or reduced glutathione, ii) at least partially reversed with ascorbate, indicative of involvement of S-nitrosylation, and iii) irreversibly blocked by low concentration of the alkylating agent, N-ethylmaleimide (NEM). The biotin-switch assay showed that both GSNO and SNAP treatments caused S-nitrosylation of TnI_f. S-glutathionylation pretreatment blocked the effects of S-nitrosylation on Ca^{2+} sensitivity, and vice-versa. S-nitrosylation pretreatment prevented NEM from irreversibly blocking S-glutathionylation of TnI_f and its effects on Ca^{2+}-sensitivity, and likewise S-glutathionylation pretreatment prevented NEM block of S-nitrosylation. Following substitution of TnI_f into rat slow-twitch fibers, S-nitrosylation treatment caused decreased Ca^{2+}-sensitivity. These findings demonstrate that S-nitrosylation and S-glutathionylation exert opposing effects on Ca^{2+}-sensitivity in mammalian FT muscle fibers, mediated by competitive actions on Cys134 of TnI_f.

Introduction

Nitric oxide (NO) is generated in skeletal muscle at rest, primarily by neuronal nitric oxide synthase (nNOS), and production increases markedly with contractile activity (5, 20, 30). Endogenous production of NO modulates submaximal force production in skeletal muscle without altering maximum force, with NOS inhibitors and NO scavengers found to increase submaximal force (20) and applied NO donors depressing force (3, 16) (for review see (30, 31, 34)). Much of the depressing effect of NO on force production is independent of cGMP and is thought to be the result of a direct action in which NO (or an NO intermediate such as GSNO) decreases the Ca^{2+}-sensitivity of the contractile apparatus. Application of NO donors S-nitroso-N-acetylcysteine and nitroprusside to intact fast-twitch muscle fibers of the mouse was found to reduce Ca^{2+}-sensitivity by ~16% (3) (i.e. decrease the pCa_{50}, the pCa at 50% force, by ~ -0.063 pCa units). It is generally presumed that NO mediates its effect by S-nitrosylation of cysteine residues on particular contractile or regulatory protein, but the protein(s) and residues involved have not been identified (31, 34). Many hundreds of cysteine residues in skeletal muscle have been found to be able to undergo S-nitrosylation, including residues on most of the contractile and associated regulatory proteins (37). Interestingly, in the mammals examined to date (rodents and rabbits) NO donors decrease Ca^{2+}-sensitivity in fast-twitch fibers (3, 11, 16) but not in slow-twitch fibers (35).

We have previously demonstrated that combined treatment with a cysteine-specific oxidant (2,2´-dithiodipyridine, DTDP) and then glutathione (GSH), or with oxidized glutathione (GSSG) alone, induces a large *increase* in Ca^{2+}-sensitivity in mammalian fast-twitch (i.e. type II) muscle fibers but not in mammalian slow-twitch (type I) fibers, nor in type II fibers from chicken or toads (21, 24, 25). The effect was evidently due to S-glutathionylation of Cys134 on fast troponin I (TnI_f) (24), which is present only in the mammalian fast-twitch fibers. The Ca^{2+}-sensitivity increase could be induced by the oxidant-GSH treatment in slow-twitch fibers after insertion of fast-twitch troponin. There are a total of four cysteine residues in the three proteins of the fast-twitch troponin complex, three on TnI_f and one on troponin C (TnC_f), but only Cys134 on TnI_f is readily accessible and reactive to oxidants in the troponin complex in-situ (8, 15) (see note in Materials and Methods on updated cysteine numbering).

In the present study we firstly directly confirm by mass spectroscopy that Cys134 on TnI_f is indeed S-glutathionylated by the oxidation-GSH treatment, and use the biotin-switch technique to show that Cys134 of TnI_f can also undergo S-nitrosylation, consistent with mass spectroscopy

102 findings (37). We consequently hypothesized that NO decreases Ca^{2+}-sensitivity in skeletal
103 muscle fibers by S-nitrosylation of Cys134 on TnI_f. We tested this by examining i) whether the
104 Ca^{2+}-sensitivity changes in various mammalian and non-mammalian muscle fibers are in accord
105 with the presence of Cys134, ii) whether S-nitrosylation and S-glutathionylation treatments show
106 competitive effects, similar sensitivity to irreversible block by N-ethylmaleamide (NEM), and a
107 reciprocal ability to protect Cys134 and the Ca^{2+}-sensitivity changes of the opposing treatment
108 from block by NEM, and iii) whether NO treatment results in reduced Ca^{2+}-sensitivity in slow-
109 twitch mammalian fibers following exchange of fast-twitch troponin. The findings provide
110 strong evidence that the NO decreases Ca^{2+}-sensitivity in skeletal muscle by inducing
111 S-nitrosylation of Cys134 on TnI_f, and that this effect competitively antagonizes the ability of
112 oxidant-induced S-glutathionylation to increase Ca^{2+}-sensitivity, which would adversely affect
113 skeletal muscle performance in various circumstances.
114

Materials and Methods

Ethical approvals and muscle fibers and muscle biopsies

All animal experiments were carried out in accordance with the Australian National Health & Medical Research Council's 'Australian code of practice for the care and use of animals for scientific purposes', and with approval of the La Trobe University Animal Ethics Committee. Male Long-Evans hooded rats (34 in total, 4 to 10 months old) and male Sprague Dawley (6 in total, 4 to 6 months old) were supplied respectively by breeding facilities at Monash University Animal House, Melbourne Victoria, and the Animal Resources Centre, Canning Vale Western Australia; there was no apparent difference between results obtained from the two rat strains (e.g. pCa shift with DTDP-GSH and GSNO treatments ~+0.22 and -0.06 pCa units, respectively, in type II fibers in both strains) nor over the age range examined. The rats were housed (2 or 3 animals per cage) in the Central Animal House of La Trobe University and kept under controlled temperature (22°C) and a 12:12 h light–dark cycle, with food and water provided *ad libitum*. They were killed by overdose of isoflurane (4 % vol./vol.) in a glass chamber, and then the EDL and soleus muscles removed by dissection. Two tropical cane toads (*Bufo marinus*) (a pest species in Australia, caught by a Queensland supplier) were maintained at 15°C to lower their activity and then stunned and killed by pithing, and the iliofibularis muscle removed. Two female chickens (~24 weeks, Hy-Line brown layer strain) from a commercial supplier were killed by overdose of intravenous phenobarbitone and a segment of pectoralis major muscle removed.

All procedures on human subjects were approved by the Human Research Ethics Committee at Victoria University. Informed consent was obtained in writing from all subjects and the studies conformed to the standards set by the Declaration of Helsinki. Skinned fiber experiments were performed with fibers from *vastus lateralis* muscle biopsies from three young male subjects (age 23 ±6 yrs; height, 174 ±9 cm; body mass, 75 ±5 kg, Mean ±SD). All subjects were healthy and participated in regular physical activity but were not specifically trained in any sport. After injection of a local anaesthetic (1% lidocaine) into the skin and fascia, a small incision was made in the middle third of the *vastus lateralis* muscle of each subject and a muscle sample taken using a Bergstrom biopsy needle by an experienced medical practioner, as described previously (22). The excised muscle sample was rapidly blotted on filter paper to remove excess blood and placed in paraffin oil (Ajax Chemicals, Sydney, Australia) for fiber dissection (see below).

Preparations and force recording

Rat and toad whole muscles, and muscle biopsies from human and chicken muscle, were pinned

149 at resting length under paraffin oil (Ajax Chemicals, Sydney, Australia) in a petri dish, and kept
150 cool (~10°C) on an icepack. Individual fiber segments were mechanically-skinned with
151 jeweller's forceps and mounted at 120 % of resting length on a force transducer (AME801,
152 SensoNor, Horten, Norway) with a resonance frequency >2 kHz. The skinned fiber segment was
153 then equilibrated for > 2 min in a perspex bath containing 2 ml of relaxing solution (see below).
154 Force responses were recorded using a Bioamp pod and Powerlab 4/20 series hardware
155 (ADInstruments, Sydney, Australia). All experiments were performed at room temperature
156 (~23 ±2 °C).
157
158 **Skinned fiber and stock solutions**
159 All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise
160 stated. As previously described (21), the 'relaxing' solution contained (in mM): $EGTA^{2-}$, 50;
161 total ATP, 8; creatine phosphate (CrP), 10; Na^{+}, 36; K^{+}, 126; total Mg^{2+}, 8.5; total; Hepes, 90; pH
162 7.1 and pCa >9. The maximum Ca^{2+}-activating solution 'max' contained 50 mM CaEGTA and
163 had a pCa ~4.7, with total Mg^{2+} adjusted to maintain 1 mM free (see (36) for apparent affinity
164 constants). These two solutions were mixed in appropriate ratio to produce solutions with pCa in
165 the range 6.7 to 4.7. All solutions had an osmolality of 295 ±5 mosmol kg^{-1}. A similar
166 strontium-based solution (with pSr = $-\log_{10}[Sr^{2+}]$ of 5.2) was made by mixing relaxing solution
167 with a Sr-EGTA solution similar to the maximum Ca^{2+}-activating solution. Exposure to a
168 solution at pSr 5.2 was used to ascertain the predominant troponin C (TnC) isoform present (see
169 (22, 24, 27, 39)). Subsequent western blotting was used to confirm the TnC isoform present in all
170 human, chicken and toad fibers and some rat fibers.
171
172 All treatments were applied in relaxing solution at pH 7.1, except for GSSG treatment which was
173 applied in relaxing solution at pH 8.5. A 100 mM stock of reduced glutathione (GSH) was made
174 in a potassium HDTA (hexa-methylene-diamine-tetraacetate) solution similar to the relaxing
175 solution but with all EGTA replaced with HDTA; the pH of the stock was re-adjusted to 7.10
176 with KOH, and then diluted 20-fold to give 5 mM in the final solution. A 100 mM GSSG stock
177 was made in relaxing solution at pH 8.5 and diluted 10-fold in the final solution. A 100 mM
178 stock solution of 2, 2'-dithiodipyridine (DTDP) was made in absolute ethanol and diluted 1000-
179 fold in the final solution to 100 µM; matching control solutions with the same amount of ethanol
180 (0.1 %) had no noticeably different effect than controls without ethanol. Similarly, NEM was
181 made as a 200 mM or 25 mM stock in ethanol and diluted 1000-fold in the final solution.
182 Dithiothreitol (DTT) was added to relaxing solution at 10 mM final concentration from a 1 M

stock made in double distilled water. Treatment with S-nitrosoglutathione (GSNO) can cause S-glutathionylation of protein thiols if applied immediately after dissolving the GSNO in solution (i.e. within 1 min) (see (11, 24)); this was specifically avoided in the present experiments and GSNO was only applied ~10 min after preparing the solution (called $GSNO_{del}$ in Dutka *et al*. (2011)), which only resulted in S-nitrosylation. SNAP (S-nitroso-N-acetyl-penicillamine, 2 or 10 mM) was added to the final solution shortly before use (within 2 min). Ascorbate was made in a 100 mM stock in relaxing solution, with the pH adjusted to 7.10, and protected from strong light during storage and application. For biotin-labelling of cysteines, following an initial 5 min wash in relaxing solution and then various treatments as specified, individual mechanically skinned EDL fiber segments were treated for 1 min in relaxing solution with 100 µM EZ-Link Biotin-HPDP (ThermoFisher Scientific), added from a 16 mM stock made in DMSO. All treatments were applied in relaxing solution (pCa >9).

Contractile apparatus experiments and analysis

The force-Ca^{2+} relationship was determined in each fiber as previously described (21, 25) by exposing the skinned fiber segment to a sequence of solutions heavily-buffered at progressively higher free [Ca^{2+}] (pCa >9 to 4.7, the latter eliciting maximum force), and then the fiber was fully relaxed again in the relaxing solution. This procedure was performed twice before ('control') and twice after each treatment to verify reproducibility and also gauge any small changes occurring with repeated activation and over time. Force produced at each [Ca^{2+}] within a given sequence was expressed relative to maximum force generated in that same sequence, and analyzed by individually fitting a Hill curve to each sequence, for each fiber segment, using GraphPad Prism 4 software, yielding separate pCa_{50} value and *h* values (pCa at half-maximum force and Hill coefficient, respectively) for every case. Maximum force reached in each force-[Ca^{2+}] sequence was expressed relative to the control level before any treatment in the given fiber, after correcting for the small decline occurring with each repetition of the force staircase (typically ~2 to 3% in EDL fibers), as gauged from the initial control repetitions in the given fiber (see also (25)). Similarly, there was a very small decrease in Ca^{2+}-sensitivity with each staircase repetition (typically ~0.0015 pCa units); this effect was adjusted for when assessing the change in Ca^{2+}-sensitivity with nitrosylation (GSNO or SNAP) or S-glutathionylation treatments, by averaging the change in pCa_{50} occurring with the treatment and that occurring when subsequently reversing the effect by DTT exposure.

216 **Dissociation of myosin and myosin light chains**

217 In order to clearly distinguish S-glutathionylation of troponin I and myosin light chain 1 (MLC1)
218 by western blotting, skinned EDL fibers were subjected to S-glutathionylation treatment by a
219 1 min exposure to 100 µM DTDP followed by a 2 min exposure to 3.5 mM biotinylated
220 glutathione ethyl ester (BioGEE, G36000, ThermoFisher Scientific), and then any remaining free
221 cysteines blocked by exposure to 5 mM NEM for 5 min (all agents applied in relaxing solution).
222 The fiber was then treated with 1% triton-X100 in relaxing solution for 5 min, washed for 5 min
223 in relaxing solution, and then placed in 10 µl of relaxing solution with 500 mM KCl for 30 min
224 ('wash solution'), in order to induce dissociation of myosin and associated myosin light chain
225 proteins out of the skinned fiber (6). The fiber was then transferred to another 10 µl aliquot of
226 the same solution and SDS added to both this solution and the wash solution, and these fiber and
227 wash samples (containing the thin filaments and structural proteins, and myosin and myosin-
228 associated proteins, respectively) were run in adjacent lanes on SDS-PAGE for western blotting.
229

230 **Western blotting**

231 Western blotting of skinned fibers was performed on the entire fiber constituents without any
232 fractionation, using non-reducing SDS-PAGE (see (24)). Each sample consisted of a single fiber
233 segment (~2 mm in length) or a group of 4 or 5 segments (~60 µg wet wt), as specified. Where
234 multiple skinned fiber segments were examined for S-glutathionylation, they were tied together
235 with a silk suture, washed in relaxing solution for at least 5 min and then transferred successively
236 to the various treatment solutions as required. After the specified treatments, each fiber sample
237 was placed in relaxing solution with 5 mM NEM for 5 min to block free sulfhydryl sites and then
238 placed in non-reducing buffer for SDS-PAGE (final concentration: 125 mM Tris pH 6.8, 10%
239 glycerol, 4% SDS, 0.01% bromophenol blue, 5 mM NEM). Proteins were separated on the
240 specified percentage SDS-PAGE gel (details provided in figure legends, Criterion TGX gels were
241 from BioRad, Hercules, CA, USA) and then wet transferred to nitrocellulose for 60 min at 100 V
242 in a circulating ice-cooled bath with transfer buffer containing 140 mM glycine, 37 mM Tris-
243 base, and 20% methanol. Membranes were then variously probed with anti-GSH (mouse
244 monoclonal, 1 in 1000, Cat No. 101-A, Virogen, Cincinnati, OH, USA); streptavidin (polyclonal
245 horse radish peroxidase (HRP), 1 in 20000, Cat No. 21140, Pierce, Thermo Fisher Scientific,
246 Australia); anti-TnI (rabbit polyclonal, 1 in 1000, Cat No. 4002, Cell Signaling Technology,
247 Danvers, MA, USA), anti-TnC (rabbit polyclonal, 1 in 400, Cat No. sc-20642, Santa Cruz
248 Biotechnology, Santa Cruz, CA, USA), anti-myosin light chain 1(mouse monoclonal, 1 in 1000,
249 Cat No. F310, Developmental Studies Hybridoma Bank (DSHB), Iowa, USA), anti-actin (rabbit

250 affinity isolated, Cat No. A2066, Sigma) all diluted in 1% bovine serum albumin in phosphate-
251 buffered saline with 0.025% Tween. Following exposure to relevant HRP secondary antibodies
252 and a series of washes in Tris-buffered saline with Tween, chemiluminescent substrate
253 (SuperSignal West Femto, Pierce) was applied to membranes and Western blot images taken with
254 ChemiDoc XRS or MP both fitted with a charge-coupled device (CCD) camera using Quantity
255 One software (Bio-Rad). With the membrane position unchanged, the white light source was
256 switched on in order to obtain an image of the pre-stained molecular weight markers on the
257 membrane.
258
259 **Biotin-switch experiments**
260 S-nitrosylation was examined using the biotin-switch technique (12, 17) (e.g. Fig. 8).
261 Each sample consisted of three EDL skinned fiber segments tied together with silk suture. Each
262 sample was subjected to a given sequence of timed treatments by transfer through a set of 0.5 ml
263 Eppendorf tubes containing relaxing solution with specific reagents, with wash periods in
264 relaxing solution between each treatment. All samples were first subjected to strong reducing
265 treatment (100 mM DTT, 10 min) in order to reverse any resting level of S-glutathionylation or
266 S-nitrosylation or other reversible oxidation states. As indicated in Figure 8, samples were then
267 S-nitrosylated by GSNO (2 mM, 10 min) or SNAP (2 mM, 10 min), with or without a preceding
268 S-glutathionylation treatment by GSSG (10 mM at pH 8.5 for 15 min) or DTDP-GSH (100 μM
269 DTDP for 5 min, followed by 5 mM GSH for 2 min). Samples were then subjected to NEM
270 blocking treatment (20 mM, 20 min), and then S-nitrosylation detected by a 12 hr exposure to
271 relaxing solution with ascorbate (50 mM, pH 7.1) and HPDP-biotin (100 μM), protected from
272 light. For a negative control, other samples were treated with DTT, then immediately blocked
273 with NEM and labelled with HPDP-biotin for 12 hr. Two positive controls were performed, one
274 where the fibers were treated with DTT and then immediately labelled with HPDP-biotin, and the
275 other where DTT (10 mM, 10 min) rather than ascorbate was used after the NEM block to reduce
276 all reversible oxidation before labelling with HPDP-biotin (e.g. Fig. 8B). Finally, all samples
277 were equilibrated for 2 min in relaxing solution with 5 mM NEM and then placed in non-
278 reducing buffer for SDS-PAGE (final concentration: 125 mM Tris pH 6.8, 10% glycerol, 4%
279 SDS, 0.01% bromophenol blue, 5 mM NEM). Proteins were then separated on 4-15% Criterion
280 Stain Free gels, wet transferred to nitrocellulose and probed for biotin with streptavidin, as above.
281

282 **Troponin exchange**

283 The troponin exchange experiments were performed on rat soleus type I fibers, as described in a
284 preceding associated study (24). The skinned fibers were first mounted on the transducer, treated
285 with Triton-X100 in relaxing solution (1% vol/vol) for 10 min and then washed in relaxing
286 solution. Troponin exchange was achieved by bathing the skinned fiber segment for 1 hr in a low
287 ionic strength rigor solution with zero Ca^{2+} and Mg^{2+} (mM: EGTA, 2.5; EDTA, 2.5; Hepes, 10,
288 pH 7.1 with KOH) with porcine fast troponin (10 mg/ml, Sigma T2275) and 2 mM DTT.

289

290 **Mass spectroscopy and proteomic analysis**

291 Proteomic experiments were performed in biological duplicate. Each sample consisted of 10
292 EDL fiber segments (~25 µg total protein) tied together with silk suture, which was
293 permeabilized with Triton-X100 for 30 min (all treatments and washes in relaxing solution),
294 washed, and treated with 10 mM DTT for 10 min, washed, and either subjected to the standard
295 S-glutathionylation treatment (100 µM DTDP for 5 min followed by 5 mM GSH for 2 min) or
296 left untreated (control). Samples were then blocked with 5 mM NEM for 2 min and placed in
297 non-reducing SDS buffer with 5 mM NEM and separated using electrophoresis (12% non-
298 reducing gel; 1.5M Tris-Cl pH 8.8, 1.5M Tris-Cl pH 6.7, 10% SDS, 30% Acrylamide
299 [Acryl:Bis = 37.5:1], 20% APS, TEMED). Individual bands corresponding to ~23 to 27 kDa
300 were excised, destained (50 mM ammonium bicarbonate/acetonitrile), alkylated (50 mM
301 iodoacetic acid for 30 min) and trypsinized (0.2 µg trypsin (Promega Sequencing Grade) for 16 h
302 at 37°C). Peptides were desalted using reverse-phase C18 StageTips, and eluted in 85% (v/v)
303 acetonitrile (ACN) in 0.5% (v/v) formic acid (FA). A nanoflow UPLC instrument (Ultimate
304 3000 RSLCnano, Thermo Fisher Scientific, Scoresby, Vic., Australia) was coupled on-line to an
305 LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion
306 source (Thermo Fisher Scientific). Peptides were loaded (Acclaim PepMap100, 5 mm × 300 µm
307 i.d., µ-Precolumn packed with 5 µm C18 beads, Thermo Fisher Scientific) and separated
308 (PepMapRSLC C18, 25 cm, 75 µm inner diameter, 2 µm 100Å, Thermo Fisher Scientific) with a
309 70-min linear gradient from 0-100% (v/v) phase B (0.1% (v/v) FA in 80% (v/v) ACN) at a flow
310 rate of 250 nL/min operated at 45 °C.

311

312 The mass spectrometer was operated in data-dependent mode where the top 20 most abundant
313 precursor ions in the survey scan (350–1500 Th) were selected for MS/MS fragmentation. Survey
314 scans were acquired at a resolution of 120,000. Unassigned precursor ion charge states and
315 singly charged species were rejected, and peptide match disabled. Maximum injection time 150

316 ms, AGC target 1×10^6, CID at 35% energy for a maximum injection time of 150 ms with AGT
317 target of 5000. Dynamic exclusion was activated for 30 s. Data were acquired using Xcalibur
318 software v2.1 (Thermo Fisher Scientific) and mgf (mascot generic file) files generated by
319 Proteome Discoverer v2.1 (Thermo Fisher Scientific).
320
321 **Database searching and protein identification**
322 Database searches were performed using Mascot 2.4 (Walter and Eliza Hall Institute Mascot
323 Server). Peptide lists were generated from a tryptic digestion with up to two missed cleavages,
324 carbamidomethylation of cysteines as fixed modifications, and as variable modifications
325 glutathione and N-ethylmaleimide. Precursor mass tolerance was 10 ppm, product ions were
326 searched at 0.6 Da tolerances, minimum peptide length defined at 6.
327
328 **Cysteine residue numbering**
329 Note that the reactive cysteine residue on TnI_f was originally numbered as Cys133 in Mollica *et*
330 *al*. (2012) in accord with earlier references (8, 40), but here it is now denoted as Cys134 to fit
331 with more recent numbering (15, 33).
332
333 **Statistics**
334 Values are presented as mean ± standard deviation (SD), with *n* denoting the number of fibers
335 examined. Statistical significance ($P<0.05$) was determined with Student's two-tailed paired
336 *t*-test.

337 **Results**

338 We have previously shown that treating mammalian fast-twitch fibers with the sulfhydryl-
339 specific agent DTDP and then with GSH, or just with oxidized GSSG alone, caused
340 S-glutathionylation of a protein with apparent molecular weight of ~25 kDa, corresponding with
341 TnI_f (e.g. see Fig. 10 in (24)). To demonstrate that this signal indeed reflected labelling of TnI_f
342 and not MLC1, which runs at a similar apparent molecular weight, these proteins were physically
343 separated by dissociating myosin and the associated light chains with a high [KCl] solution, and
344 then the dissociated proteins and those remaining in the fiber (i.e. the thin filaments and structural
345 proteins) were run in adjacent lanes on SDS-PAGE for western blotting (see Materials and
346 Methods). This separation clearly showed that the DTDP-GSH treatment used here, which
347 elicited a large increase in Ca^{2+}-sensitivity, produced marked S-glutathionylation of TnI_f and not
348 of MLC1 (Fig. 1). Furthermore, mass spectroscopy of control and treated muscle fiber samples
349 directly demonstrated that the DTDP-GSH treatment produced S-glutathionylation of Cys134 on
350 TnI_f (Fig. 2). No S-glutathionylation of Cys134 was found in the control samples, whereas the
351 tryptic fragments containing Cys134 identified in the DTDP-GSH treated samples had all
352 undergone S-glutathionylation, and interestingly were cleaved at Lys130 and not at Lys132 as
353 found for the fragments in the control samples (see Discussion).
354

355 **Effects of S-nitrosylation treatments**

356 In accord with previous observations (11, 35), treatment of rat skinned fast twitch (type II)
357 fibers with the S-nitrosylating agents GSNO or SNAP caused a decrease in Ca^{2+}-sensitivity by
358 ~-0.06 to -0.08 pCa units with little or no change in maximum Ca^{2+}-activated force (e.g. Fig. 3)
359 (Tables 1 & 2). Similar effects were seen when treating fibers with a relatively low concentration
360 of GSNO for a prolonged time (200 μM GSNO for 30 min) or with a higher concentration for a
361 shorter time (2 mM for 2 min) (Table 1). The effect on Ca^{2+}-sensitivity was largely or fully
362 reversed by a 10 min exposure to 10 mM DTT, indicating involvement of cysteine residues, and
363 also partially reversed by a 10 min exposure to ascorbate (2 to 50 mM) indicating the effects were
364 due at least in part to S-nitrosylation of the cysteine residues (12); the mean ±SD increase in
365 pCa_{50} upon ascorbate reversal was +0.044 ±0.004, n=4 for GSNO-treated fibers and
366 +0.079 ±0.014, n=3 for SNAP-treated fibers, which corresponded to 59 ±14% and 65 ±7%
367 respectively of the reversal found with DTT treatment in the same fibers (e.g. Fig. 3). Ascorbate
368 treatment had no effect on Ca^{2+}-sensitivity in fibers that had already been reduced by DTT
369 (change in pCa_{50}: -0.009 ± 0.009, n=4).
370

As reported by Spencer & Posterino (35), the S-nitrosylation treatments had no significant effect on Ca^{2+}-sensitivity in rat type I (slow-twitch) fibers (Table 1). Similarly, we found that in human *vastus lateralis* muscle fibers, SNAP treatment caused a decrease in Ca^{2+} sensitivity in type II (fast-twitch) fibers (change in $pCa_{50:}$ -0.090 ±0.018, n=10) and not in type I fibers (-0.009 ±0.006, n=3). In contrast, SNAP treatment had no effect on the Ca^{2+}-sensitivity in type II fibers of either chicken or cane toad (Table 1). These findings are highly analogous to the effects of S-glutathionylation treatment, which was found to affect (*increase* not decrease) Ca^{2+}-sensitivity in mammalian type II fibers and not in mammalian type I fibers nor in chicken or toad type II fibers; those effects were the result of S-glutathionylation of Cys134 on troponin I (24), which is present only in mammalian type II fibers ((15, 40). To further explore this parallel, we examined whether the S-nitrosylation and S-glutathionylation treatments displayed competitive actions, which would be suggestive of a common site of action.

Competitive effects of S-nitrosylation and S-glutathionylation treatments

After a rat type II fiber had been subjected to S-glutathiolynation treatment with GSSG, which increased Ca^{2+}-sensitivity (e.g. Fig. 4), S-nitrosylation treatment with GSNO caused no further change in Ca^{2+}-sensitivity (mean change in pCa_{50} +0.004 ±0.004 pCa units, n=3), but following subsequent reversal of the S-glutathiolynation with DTT, GSNO treatment once again elicited a substantial decrease in Ca^{2+}-sensitivity (-0.073 ±0.002, n=3). A similar blocking effect was seen irrespective of whether GSSG or DTDP-GSH treatment was used as the S-glutathionylating pre-treatment, or whether GSNO or SNAP treatment was used to elicit S-nitrosylation (data not shown).

Conversely, in rat type II fibers that had undergone S-nitrosylation treatment with SNAP (10 mM, 2 min), subsequent S-glutathionylation treatment with DTDP-GSH resulted in the Ca^{2+}-sensitivity increasing to only a small net amount above the original starting level (final level +0.041 ±0.001 pCa units, n=3), which was only ~16% of the increase in Ca^{2+} sensitivity produced by the same S-glutathionylation treatment when the fibers had not been pre-treated with SNAP. This indicates that the S-nitrosylation pre-treatment with SNAP was sufficient to block most of the effect on Ca^{2+}-sensitivity of this strong S-glutathionylation treatment (see (21, 24). Furthermore, western blotting of skinned EDL fibers for anti-GSH directly showed that S-nitrosylating pre-treatment with SNAP largely prevented S-gluthionylation of TnI_f (e.g. Fig. 5A).

403 **Block by NEM**

404 The preceding experiments indicated that the S-glutathionylation and S-nitrosylation
405 treatments each largely blocked the action of the other treatment on Ca^{2+}-sensitivity. To further
406 test whether this was because the two treatments both targetted the same cysteine residue(s), we
407 examined the blocking action of N-ethylmaleimide (NEM) on each of the processes. We have
408 previously shown that a 2 min application of a low concentration (25 μM) of the alkylating agent
409 NEM largely prevents S-glutathionylation of TnI_f, irreversibly blocking >85% of the normal
410 Ca^{2+}-sensitivity increase (24). We found here that the same NEM treatment (25 μM for 2 min)
411 also blocked ~90% of the Ca^{2+}-sensitivity shift to S-nitrosylation treatment by either GSNO or
412 SNAP (right-hand column for treatments 3 to 6 in Table 2). Importantly also, S-nitrosylation pre-
413 treatment prevented NEM from irreversibly blocking the effects of a subsequent
414 S-glutathionylation treatment (applied after DTT reversal of the effects of the initial
415 S-nitrosylation) (e.g. Fig. 6 A & B), and conversely, pre-treatment by S-glutathionylation
416 prevented NEM from blocking the effects of S-nitrosylation (e.g. Fig. 6C), irrespective of which
417 specific S-nitrosylation and S-glutathionylation treatments were used (see summarized data in
418 Table 2).

419

420 Furthermore, western blotting of rat type II fibers with anti-GSH directly showed that NEM
421 blocked S-glutathionylation of TnI_f (Fig. 5B and also see (24)) and that pre-treatment with SNAP
422 prevented such NEM block (Fig. 5B). Moreover, NEM treatment blocked the ability of biotin-
423 HPDP (a biotin tagged analogue of DTDP) to react with and label TnI_f at Cys134, and pre-
424 treatment with SNAP prevented this blocking action of NEM (e.g. Fig. 7). These data
425 demonstrate that SNAP and NEM both competitively target the same cysteine residue on TnI that
426 undergoes S-glutathionylation, namely Cys134.

427

428 **Biotin-switch assay for S-nitrosylation**

429 The biotin-switch technique (12, 17) was used to directly identify proteins in the rat type
430 II muscle fibers undergoing S-nitrosylation with the GSNO and SNAP treatments. This showed
431 that both treatments caused significant S-nitrosylation of TnI_f, as well as S-nitrosylation of the
432 fast isoform of myosin light chain 1 (MLC1) (seen at ~25 kDa) and myosin light chain 3 (MLC3)
433 (seen at ~16 kDa) (see Fig. 8A), and also another protein running at ~140 kDa which was
434 probably myosin binding protein C (MyBPC) (not shown) (see (37)). Furthermore, it was found
435 that the 10 min treatment with 2 mM GSNO produced a comparable level of S-nitrosylation of
436 TnI_f as that seen with the positive control treatment in which fibers were exposed to the biotin

437 labelling agent without any pre-treatment with the S-nitrosylating and reducing agents (ascorbate
438 or DTT) (e.g. Fig. 8B). This showed that the standard GSNO treatment produced very substantial
439 S-nitrosylation of TnI_f. Furthermore, S-glutathionylation pretreatment of the fibers with either
440 GSSG or DTDP-GSH was found to markedly block the ability of the GSNO treatment to produce
441 S-nitrosylation of TnI_f (see Fig. 8B).
442
443 **GSH treatment simply reverses S-nitrosylation of TnI_f**
444 Given that Cys134 on TnI_f evidently can undergo either S-glutathionylation or
445 S-nitrosylation, with opposing effects on Ca^{2+}-sensitivity of the contractile apparatus, an
446 important further question was whether exposure of the S-nitrosylated residue (i.e. RSNO) to
447 reduced glutathione (GSH) would result in it being reduced back to a free sulphydryl (i.e. RSH)
448 or instead becoming S-glutathionylated (i.e. RSSG). This was examined by first S-nitrosylating a
449 rat type II fiber with SNAP or GSNO and then examining the effects of two successive 2 min
450 exposures to GSH (5 mM). The GSNO treatment decreased Ca^{2+}-sensitivity by -0.090 ±0.016
451 pCa units (n=4) and the first GSH exposure resulted in the Ca^{2+}-sensitivity shifting back to its
452 original level (+0.002 ±0.003 pCa units relative to original control level) and the second GSH
453 exposure caused no further change (+0.005 ±0.007 pCa units relative to original). The GSH
454 exposures following S-nitrosylation with SNAP had similar effect (final level: +0.004 ±0.005 and
455 -0.001 ±0.04 pCa units relative to original level, n=3). Given that it takes a very prolonged
456 exposure to GSH (>20 min) to even partially reverse S-glutathionylation of TnI_f (21), it can be
457 concluded that when Cys134 on TnI_f is S-nitrosylated, exposure to GSH simply reduces it back to
458 a free sulphydryl rather than causes it to undergo S-glutathionylation. It was also verified that
459 Cys134 had been converted back to its reduced state by showing subsequent DTDP-GSH
460 treatment elicited the normal S-glutathionylation effect on Ca^{2+}-sensitivity. In two further cases,
461 fibers were S-nitrosylated with SNAP and then exposed to 10 mM GSSG at normal pH (7.1),
462 which had no effect on the Ca^{2+}-sensitivity, indicating that Cys134 remained in its S-nitrosylated
463 state and GSSG did not cause S-glutathionylation.
464
465 **Effect of troponin exchange**
466 Finally, we examined whether exchanging the troponin complex in rat slow-twitch
467 (type 1) muscle fibers with fast-twitch troponin affected the response of the fibers to
468 S-nitrosylation treatment with SNAP. These experiments were done as part of the troponin
469 exchange studies detailed in Mollica *et al*. (24), where ~35% of the TnI in the soleus fibers was
470 exchanged with TnI_f and which resulted in the type I fibers showing increased Ca^{2+}-sensitivity

upon S-glutathionylation treatment (~+0.13 pCa units versus no change before exchange). After such partial exchange with TnI_f, S-nitrosylation treatment with SNAP (10 mM, 2 min) resulted in a significant decrease in Ca^{2+}-sensitivity in the three type I fibers examined (pCa_{50} decreasing by -0.026 ± 0.011 pCa units, compared to -0.001 ±0.002 pCa units in untreated fibers, Table 1); this sample included the fiber shown in Fig. 7 of Mollica *et al*. (24) that displayed increased Ca^{2+}-sensitivity to S-glutathionylation treatment after TnI_f exchange, as well as a fiber in which SNAP treatment was also tested before the TnI_f exchange and found to have no effect on Ca^{2+}-sensitivity (zero change in pCa_{50}).

Discussion

The findings of this study provide compelling evidence that the action of NO in decreasing Ca^{2+}-sensitivity in skeletal muscle is mediated by S-nitrosylation of Cys134 on TnI_f. The evidence for this is as follows: i) Cys134 on TnI_f in mammalian fast-twitch muscle undergoes S-glutathionylation and S-nitrosylation respectively when muscle fibers are subjected to the specific S-glutathionylation treatments (DTDP-GSH or GGSG at pH 8.5) and S-nitrosylation treatments (GSNO or SNAP) used here (see mass spectroscopy results here in Fig. 2 and in Su *et al.* (2013) and Fig.s 1 & 8); ii) the decrease in Ca^{2+}-sensitivity with S-nitrosylation treatment is seen only in mammalian fast-twitch (i.e. type II) fibers (e.g. rat, human and rabbit), and not in mammalian slow-twitch (i.e. type I) fibers nor in toad or chicken type II fibers (see Results and Table 1), in accord with the presence of Cys134 on TnI_f; iii) the effects of S-nitrosylation treatment can be reversed with ascorbate (Fig. 3), a specific reversal agent (12); iv) S-glutathionylation treatment in mammalian fast-twitch fibers blocks S-nitrosylation of TnI_f (Fig. 8) and its effect on Ca^{2+}-sensitivity (Fig. 4), and S-nitrosylation treatment blocks S-glutathionylation of TnI_f (Fig. 5A) and its effects on Ca^{2+}-sensitivity (see Results); v) NEM irreversibly blocks both S-glutathionylation and S-nitrosylation with very similar efficacy (~90% block by 20 μM NEM for 2 min) (Table 2 and (24)); vi) S-nitrosylation pre-treatment prevents NEM from blocking S-glutathionylation of TnI_f (Fig. 5B & 7) and its effect on Ca^{2+}-sensitivity (Fig. 6A & B), and conversely S-glutathionylation pre-treatment prevents NEM from blocking the effects of S-nitrosylation (Figure 6C); and finally, vii) S-nitrosylation treatment decreases Ca^{2+}-sensitivity in rat slow-twitch fibers after exchanging in fast-twitch troponin (see Results).

A substantial proportion of cysteine residues in many proteins, including in skeletal muscle (37), can undergo S-nitrosylation, but fewer cysteines residues seemingly can undergo S-glutathionylation, and only a small subset are able to undergo both types of modification (4, 13, 14). The findings here indicate that Cys134 on TnI_f is able to be S-glutathionylated or S-nitrosylated, and that these alternate modifications of the one cysteine residue have opposing functional effects, increasing or decreasing the Ca^{2+}-sensitivity of contractile apparatus respectively (e.g. Fig. 4). Cys134 is in the flexible and highly mobile C-terminal domain of TnI_f, immediately adjacent to the 'switch' region that binds to the hydrophobic pocket in the N-lobe of TnC in the Ca^{2+}-bound state and swings back to be frequently near actin in the absence of Ca^{2+} (1, 32). S-glutathionylation effectively adds a negative charge at a cysteine residue, which together with the accompanying steric effects, is thought to exert an action similar to that occurring with protein phosphorylation (9, 19). It seems that the net effect of S-glutathionylation

515 at Cys134 is to bias the movements of the switch region towards the TnC bound state, so that the
516 interaction between TnI and TnC controlling contractile activation occurs at lower cytoplasmic
517 [Ca^{2+}]. The effect of S-glutathionylation of Cys134 in altering TnI_f's tertiary structure is also
518 directly indicated by the slower migration of the S-glutathionylated protein on SDS-PAGE (24)
519 and the reduced susceptibility to trypsin cleavage of the nearby Lys132 residue, as apparent in
520 our mass spectroscopy data (Fig. 2). S-nitrosylation of Cys134, on the other hand, *decreases* the
521 Ca^{2+}-sensitivity of contractile activation, indicating that its overall effect presumably is to bias the
522 movements of the switch region of TnI_f *away* from TnC.
523
524 In the native troponin complex in-situ, Cys134 on TnI_f is readily accessible and reactive both in
525 the presence and absence of Ca^{2+} (8, 15), and hence readily susceptible to either
526 S-glutathionylation or S-nitrosylation. The mass spectroscopy study of Su *et al*. (37) found that
527 the GSNO treatment in mouse muscle homogenates also resulted in S-nitrosylation of Cys49 and
528 Cys65 on TnI_f and of Cys99 on TnC_f. However, S-nitrosylation of these residues likely only
529 occurred because the muscle homogenate was treated in a low ionic strength solution in the
530 absence of any Ca^{2+} and Mg^{2+}, conditions which induce dissociation of the troponin complex (see
531 (24)), because all these three cysteine residues are normally inaccessible to modification in the
532 native troponin complex (8, 15).
533
534 Given that S-glutathionylation and S-nitrosylation of Cys134 have opposing functional effects, it
535 was important to examine whether the millimolar levels of reduced GSH normally present in
536 rested muscle fibers (18) might be expected to cause any S-nitrosylated residues to undergo
537 S-glutathionylation (i.e. RSNO + GSH → RSSG + HNO) (see (13, 19)). It was instead found
538 that the presence of GSH evidently converted S-nitrosylated Cys134 residues back to their
539 reduced state (i.e. RSNO + GSH → RSH + GSNO), simply reversing the decrease in Ca^{2+}-
540 sensitivity rather than inducing a marked increase. It is interesting to relate this to the findings of
541 Andrade *et al*. (3), where application of the NO donor S-nitroso-N-acetylcysteine (SNAC) to
542 intact fast-twitch fibers of the mouse decreased Ca^{2+}-sensitivity by ~ -0.065 pCa units, which was
543 reversed within 1 min simply by washout of the SNAC without application of any specific
544 reducing treatment. In the skinned fibers examined in the present study, sensitivity changes
545 elicited by S-nitrosylation or S-glutathionylation treatments remained unchanged indefinitely
546 until specifically reversed by application of a reducing treatment, such as DTT or GSH. It seems
547 that in the intact fiber experiments of Andrade et al (3), the presence of extracellular SNAC gave
548 rise to a steady influx of NO, resulting directly or indirectly in S-nitrosylation of Cys134 on TnI_f,

549 and that when the NO influx ceased upon washing away the SNAC, the remaining level of
550 reduced GSH present in the fiber was sufficient to quite rapidly reverse the S-nitrosylation of
551 TnI_f. (It is possible that the reversal of the S-nitrosylation effects in the experiments of Andrade
552 *et al.* (3) might also have been aided by hemolysis of the RS-NO bond by the ultraviolet light
553 used for imaging intracellular Ca^{2+} in those fibers).
554
555 The above findings together offer important insight into the possible actions of NO in skeletal
556 muscle fibers, indicating that if NO generation within a fiber were to markedly increase in some
557 situation, it could be expected to readily target Cys134 on TnI_f, not only directly decreasing Ca^{2+}-
558 sensitivity but also blocking any Ca^{2+}-sensitivity increase to S-glutathionylation, negating its
559 beneficial effects in exercising muscle (2, 24). If the increase in NO were relatively small and
560 brief, its inhibitory effects on Ca^{2+}-sensitivity would probably be only transient, being quickly
561 reversed by the normal reducing environment within the muscle fiber, whereas if the increase in
562 NO were very large or prolonged it presumably would also perturb the redox environment of the
563 fiber and the inhibitory effects of the NO may be long-lasting. However, if the increase in NO
564 were preceded by an increase in reactive oxygen species, it is quite likely that Cys134 would
565 have already undergone S-glutathionylation, and the Ca^{2+}-sensitivity would be heightened and
566 remain unaffected by the NO, particularly given that the reversal of S-glutathionylation of
567 Cys134 is relatively slow (21).
568
569 The overall importance of NO's direct effects on muscle force is currently unclear and likely
570 differs considerably in various conditions. NO is generated in normal resting skeletal muscle and
571 its production increases with muscular activity (5, 20, 30). Current data are equivocal as to
572 whether the direct inhibitory effects of NO are a significant factor in normal muscle fatigue (30,
573 34), but this is a complicated question because NO not only has direct inhibitory effects on
574 contractile function but it also has stimulatory effects on Ca^{2+} release from the sarcoplasmic
575 reticulum (3, 29) and on the blood supply to the muscle. Furthermore, high or very prolonged
576 levels of NO may not only decrease Ca^{2+}-sensitivity but also reduce maximum force production
577 of the contractile proteins by also acting on the myosin heads (26), including via the production
578 and action of peroxynitrite (10, 38). Such inhibitory actions of NO are believed to play a major
579 role in the decreased muscle function in hypoxia (28, 41) and in sepsis (7, 23). The levels of NO
580 and GSNO applied in the present study were likely very much higher than the levels reached
581 in-vivo, but they were applied for only relatively brief periods, and because these nitrosylating
582 treatments caused little or no decrease in maximum force production it is apparent that they

583 elicited reversible physiological alterations and not the irreversible pathological modifications of
584 the contractile apparatus that occur in certain circumstances.
585
586 **Conclusions**
587 This study provides evidence that the direct inhibitory effect of nitric oxide on Ca^{2+}-sensitivity in
588 skeletal muscle is due to S-nitrosylation of Cys134 on TnI_f. Significantly, this same site can
589 undergo S-glutathionylation in the presence of oxidants and glutathione, which has the opposite
590 functional effect, increasing the Ca^{2+}-sensitivity of muscle contraction. Production of nitric oxide
591 and reactive oxygen species both increase with muscle activation, as well as in hypoxia and in
592 particular pathological conditions. Both S-nitrosylation and S-glutathionylation can have
593 important protective effects, preventing irreversible oxidative damage (e.g. sulphonation) of key
594 cysteine residues. S-glutathionylation of TnI_f has been observed to occur in human muscle after
595 prolonged cycling (24), and would be expected to be beneficial to muscle performance by
596 compensating to some extent for the actions of the many metabolic factors that decrease
597 contractile Ca^{2+}-sensitivity with exercise (2). Given its opposing competitive effects, the extent
598 of S-nitrosylation of Cys134 remains an important but unresolved issue, with its relative role
599 likely being greater in specific exercise and disease conditions.
600

601 **Author contributions**

602 TLD performed physiological experiments and analyzed related data. JPM and RMM were
603 responsible the biochemical experiments and related analyses. CRL recruited human subjects
604 and organized biopsy samples and related single fiber experiments. VCW and DWG were
605 responsible for the mass spectroscopy experiments. GSP helped conceive and design the
606 physiology experiments. GDL conceived and designed and helped analyze most experiments and
607 drafted the manuscript with input from all authors. All authors have read and approved the final
608 version of the submitted manuscript and attest that all persons designated as authors qualify for
609 authorship, and all those who qualify for authorship are listed. The experiments were performed
610 at La Trobe University (Melbourne, Victoria, Australia).
611
612
613 **Competing interests and Funding**
614 The authors declare that they have no competing interests in regard to this study. We thank the
615 National Health & Medical Research Council of Australia for financial support (Grant number
616 1051460).
617
618
619
620 **Acknowledgments**
621 We thank Maria Cellini, Heidy Flores and Barney Frankish for technical assistance. Proteomics
622 in this study was supported using the Mass Spectrometry and Proteomics facility, La Trobe
623 University. DWG was supported by the LIMS Molecular Biology Stone Fellowship, La Trobe
624 University Research Focus Area Leadership Grant, and La Trobe University Start-up Fund.
625 Proteomics in this study was supported using the Mass Spectrometry and Proteomics facility, La
626 Trobe University.
627
628

References

1. **Aihara T, Nakamura M, Ueki S, Hara H, Miki M, and Arata T**. Switch action of troponin on muscle thin filament as revealed by spin labeling and pulsed EPR. *J Biol Chem* 285: 10671-10677, 2010.
2. **Allen DG, Lamb GD, and Westerblad H**. Skeletal muscle fatigue: cellular mechanisms. *Physiol Rev* 88: 287–332, 2008.
3. **Andrade FH, Reid MB, Allen DG, and Westerblad H**. Effect of nitric oxide on single skeletal muscle fibres from the mouse. *J Physiol* 509 577-586, 1998.
4. **Aracena-Parks P, Goonasekera SA, Gilman CP, Dirksen RT, Hidalgo C, and Hamilton SL**. Identification of cysteines involved in S-nitrosylation, S-glutathionylation, and oxidation to disulfides in ryanodine receptor type 1. *J Biol Chem* 281: 40354-40368, 2006.
5. **Balon TW, and Nadler JL**. Nitric oxide release is present from incubated skeletal muscle preparations. *J Appl Physiol (1985)* 77: 2519-2521, 1994.
6. **Bicer S, and Reiser PJ**. Variations in apparent mass of mammalian fast-type myosin light chains correlate with species body size, from shrew to elephant. *Am J Physiol Regul Integr Comp Physiol* 292: R527-534, 2007.
7. **Callahan LA, Nethery D, Stofan D, DiMarco A, and Supinski G**. Free radical-induced contractile protein dysfunction in endotoxin-induced sepsis. *American journal of respiratory cell and molecular biology* 24: 210-217, 2001.
8. **Chong PC, and Hodges RS**. Proximity of sulfhydryl groups to the sites of interaction between components of the troponin complex from rabbit skeletal muscle. *J Biol Chem* 257: 2549-2555, 1982.
9. **Dalle-Donne I, Rossi R, Giustarini D, Colombo R, and Milzani A**. S-glutathionylation in protein redox regulation. *Free Radic Biol Med* 43: 883-898, 2007.
10. **Dutka TL, Mollica JP, and Lamb GD**. Differential effects of peroxynitrite on contractile protein properties in fast- and slow-twitch skeletal muscle fibers of rat. *J Appl Physiol* 110: 705-716, 2011.
11. **Dutka TL, Mollica JP, Posterino GS, and Lamb GD**. Modulation of contractile apparatus Ca2+ sensitivity and disruption of excitation-contraction coupling by S-nitrosoglutathione in rat muscle fibres. *J Physiol* 589: 2181-2196, 2011.
12. **Forrester MT, Foster MW, Benhar M, and Stamler JS**. Detection of protein S-nitrosylation with the biotin-switch technique. *Free Radic Biol Med* 46: 119-126, 2009.
13. **Giustarini D, Milzani A, Aldini G, Carini M, Rossi R, and Dalle-Donne I**. S-nitrosation versus S-glutathionylation of protein sulfhydryl groups by S-nitrosoglutathione. *Antioxidants & redox signaling* 7: 930-939, 2005.
14. **Gould N, Doulias PT, Tenopoulou M, Raju K, and Ischiropoulos H**. Regulation of protein function and signaling by reversible cysteine S-nitrosylation. *J Biol Chem* 288: 26473-26479, 2013.
15. **Gross SM, and Lehman SL**. Accessibility of myofilament cysteines and effects on ATPase depend on the activation state during exposure to oxidants. *PloS one* 8: e69110, 2013.
16. **Heunks LM, Cody MJ, Geiger PC, Dekhuijzen PN, and Sieck GC**. Nitric oxide impairs Ca2+ activation and slows cross-bridge cycling kinetics in skeletal muscle. *J Appl Physiol* 91: 2233-2239, 2001.
17. **Jaffrey SR, and Snyder SH**. The biotin switch method for the detection of S-nitrosylated proteins. *Science's STKE : signal transduction knowledge environment* 2001: pl1, 2001.
18. **Ji LL, Fu R, and Mitchell EW**. Glutathione and antioxidant enzymes in skeletal muscle: effects of fiber type and exercise intensity. *J Appl Physiol* 73: 1854-1859, 1992.
19. **Klatt P, and Lamas S**. Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. *Eur J Biochem* 267: 4928-4944, 2000.

20. **Kobzik L, Reid MB, Bredt DS, and Stamler JS**. Nitric oxide in skeletal muscle *Nature* 372: 546-548, 1994.

21. **Lamb GD, and Posterino GS**. Effects of oxidation and reduction on contractile function in skeletal muscle fibres of the rat. *J Physiol* 546: 149-163, 2003.

22. **Lamboley CR, Murphy RM, McKenna MJ, and Lamb GD**. Endogenous and maximal sarcoplasmic reticulum calcium content and calsequestrin expression in Type I and Type II human skeletal muscle fibres. *J Physiol* 591: 6053–6068, 2013.

23. **Lin MC, Ebihara S, El Dwairi Q, Hussain SN, Yang L, Gottfried SB, Comtois A, and Petrof BJ**. Diaphragm sarcolemmal injury is induced by sepsis and alleviated by nitric oxide synthase inhibition. *American journal of respiratory and critical care medicine* 158: 1656-1663, 1998.

24. **Mollica JP, Dutka TL, Merry T, Lamboley C, McConell GK, McKenna MJ, Murphy RM, and Lamb GD**. S-glutathionylation of Troponin I (fast) increases contractile apparatus Ca2+-sensitivity in fast-twitch muscle fibres of rats and humans. *J Physiol* 590: 1443-1463, 2012.

25. **Murphy RM, Dutka TL, and Lamb GD**. Hydroxyl radical and glutathione interactions alter calcium sensitivity and maximum force of the contractile apparatus in rat skeletal muscle fibres. *J Physiol* 586: 2203-2216, 2008.

26. **Nogueira L, Figueiredo-Freitas C, Casimiro-Lopes G, Magdesian MH, Assreuy J, and Sorenson MM**. Myosin is reversibly inhibited by S-nitrosylation. *Biochem J* 424: 221-231, 2009.

27. **O'Connell B, Nguyen LT, and Stephenson GM**. A single-fibre study of the relationship between MHC and TnC isoform composition in rat skeletal muscle. *Biochem J* 378: 269-274, 2004.

28. **Ottenheijm CA, Heunks LM, Geraedts MC, and Dekhuijzen PN**. Hypoxia-induced skeletal muscle fiber dysfunction: role for reactive nitrogen species. *American journal of physiology* 290: L127-135, 2006.

29. **Pouvreau S, Allard B, Berthier C, and Jacquemond V**. Control of intracellular calcium in the presence of nitric oxide donors in isolated skeletal muscle fibres from mouse. *J Physiol* 560: 779-794, 2004.

30. **Powers SK, and Jackson MJ**. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev* 88: 1243-1276, 2008.

31. **Powers SK, Ji LL, Kavazis AN, and Jackson MJ**. Reactive oxygen species: impact on skeletal muscle. *Comprehensive Physiology* 1: 941-969, 2011.

32. **Sheng JJ, and Jin JP**. TNNI1, TNNI2 and TNNI3: Evolution, regulation, and protein structure-function relationships. *Gene* 576: 385-394, 2016.

33. **Sheng Z, Pan BS, Miller TE, and Potter JD**. Isolation, expression, and mutation of a rabbit skeletal muscle cDNA clone for troponin I. The role of the NH2 terminus of fast skeletal muscle troponin I in its biological activity. *J Biol Chem* 267: 25407-25413, 1992.

34. **Smith MA, and Reid MB**. Redox modulation of contractile function in respiratory and limb skeletal muscle. *Respiratory physiology & neurobiology* 151: 229-241, 2006.

35. **Spencer T, and Posterino GS**. Sequential effects of GSNO and H2O2 on the Ca2+ sensitivity of the contractile apparatus of fast- and slow-twitch skeletal muscle fibers from the rat. *Am J Physiol Cell Physiol* 296: C1015-1023, 2009.

36. **Stephenson DG, and Williams DA**. Calcium-activated force responses in fast- and slow-twitch skinned muscle fibres of the rat at different temperatures. *J Physiol* 317: 281-302, 1981.

37. **Su D, Shukla AK, Chen B, Kim JS, Nakayasu E, Qu Y, Aryal U, Weitz K, Clauss TR, Monroe ME, Camp DG, 2nd, Bigelow DJ, Smith RD, Kulkarni RN, and Qian WJ**. Quantitative site-specific reactivity profiling of S-nitrosylation in mouse skeletal muscle using cysteinyl peptide enrichment coupled with mass spectrometry. *Free Radic Biol Med* 57: 68-78, 2013.

38. **Supinski G, Stofan D, Callahan LA, Nethery D, Nosek TM, and DiMarco A**. Peroxynitrite induces contractile dysfunction and lipid peroxidation in the diaphragm. *J Appl Physiol* 87: 783-791, 1999.

39. **Trinh HH, and Lamb GD**. Matching of sarcoplasmic reticulum and contractile properties in rat fast- and slow-twitch muscle fibres. *Clin Exp Pharmacol Physiol* 33: 591-600, 2006.

40. **Wilkinson JM, and Grand RJ**. Comparison of amino acid sequence of troponin I from different striated muscles. *Nature* 271: 31-35, 1978.

41. **Zhu X, Heunks LM, Ennen L, Machiels HA, and Dekhuijzen PN**. Role of nitric oxide in isometric contraction properties of rat diaphragm during hypoxia. *Eur J Appl Physiol* 88: 417-426, 2003.

742
743 Table 1. Effect of S-nitrosylation treatments on Ca^{2+}-sensitivity in different types of muscle fibres. Mean (±SD) change in pCa_{50} in given type of
744 muscle fibre after treatment with SNAP (10 mM, 2 min) or GSNO (2 mM, 2 min; 200 μM, 30 min). Type II (fast-twitch) fibres obtained from
745 EDL muscle in rat, vastus lateralis muscle in humans, pectoralis muscle in chicken, and from twitch portion of iliofibularis muscle in toad, and
746 type I (slow-twitch) fibres obtained from soleus muscle in rat and vastus lateralis muscle in humans. Number of fibres given in brackets. Value
747 for each individual fibre ascertained from the pCa change upon treatment and upon its subsequent reversal by DTT treatment (10 mM, 10 min), so
748 as to remove the effect of the small progressive decrease in pCa_{50} occurring with each successive force-pCa staircase. * denotes significantly
749 different from zero ($P<0.05$, Student's two-tailed t-test).
750

Treatment	Rat type II	Rat type I	Human type II	Human type I	Toad type II	Chicken type II
SNAP	-0.057 ±0.011 (4) *	-0.001 ±0.002 (3)	-0.090 ±0.018 (10) *	-0.009 ±0.006 (3)	+0.004 ±0.003 (5)	+0.001 ±0.003 (4)
GSNO (2 mM, 2 min)	-0.063 ±0.010 (7) *	-0.008 ±0.009 (6)				
GSNO (200 μM, 30 min)	-0.064 ±0.011 (3) *					

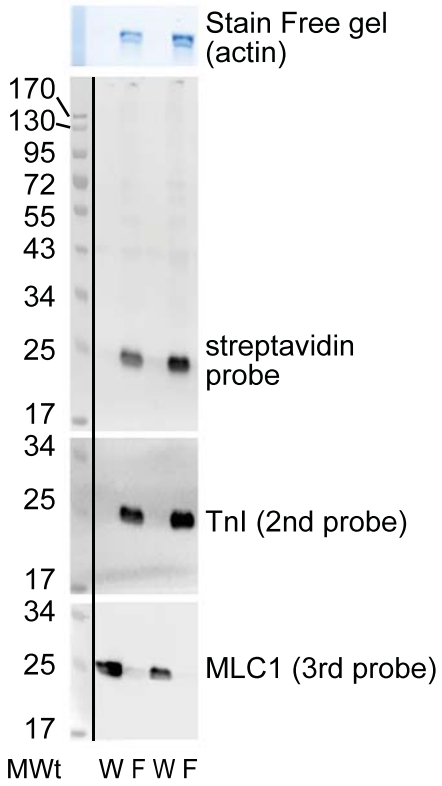
764 Table 2. S-nitrosylation and S-glutathionylation treatments bestow reciprocal protection from block by NEM. Mean (±SD) of pCa_{50} shift in rat
765 type II fibres to S-glutathionylation treatment (cases 1 & 2) or S-nitrosylation treatment (cases 3 to 6) when performed on its own, or after
766 pretreatment with opposing treatment and then NEM, or after pretreatment with NEM alone (see Fig. 5). Number of fibres (n).
767 S-glutathionylation treatments: i) DTDP-GSH (i.e. 100 µM DTDP for 5 min followed by 5 mM GSH for 2 min) or ii) GSSG (10 mM at pH 8.5 for
768 10 min). S-nitrosylation treatments: i) GSNO (2 mM, 10 min) or ii) SNAP (10 mM, 2 min). NEM blocking treatment: 25 µM NEM for 2 min.
769 Percentage change relative to that for treatment alone (i.e. without any pretreatments) shown in brackets. # Case of GSSG treatment alone not
770 examined here and mean value shown is that reported previously in Mollica et al. (2012), and the corresponding percentage changes with
771 pretreatments are only approximate.
772
773
774

Treatment alone		With Indicated pretreatments		With NEM pretreatment alone
1) DTDP-GSH (n=3)	**+0.224 ±0.011 (100%)**	**GSNO, then NEM:**	**+0.219 ±0.003 (98 ±1%)**	**+0.035 ±0.006 (15 ±2%)**
2) GSSG (n=3)	**+0.191 ±0.022 #**	**SNAP, then NEM:**	**+0.194 ±0.017 (~ 100%)**	**-0.010 ±0.013 (~ 0%)**
3) GSNO (n=3)	**-0.062 ±0.004 (100%)**	**GSSG, then NEM:**	**-0.057 ±0.009 (91 ±15%)**	**-0.007 ±0.0005 (11 ±1%)**
4) GSNO (n=4)	**-0.085 ±0.024 (100%)**	**DTDP-GSH, then NEM: (1 min in DTDP)**	**-0.085 ±0.012 (100 ±12%)**	**- 0.009 ±0.004 (9 ±4%)**
5) GSNO (n=3)	**-0.059 ±0.005 (100%)**	**DTDP-GSH, then NEM: (5 min in DTDP)**	**-0.064 ±0.005 (109 ±8%)**	**- 0.006 ±0.001 (10 ±2%)**
6) SNAP (n=2)	**-0.057 ±0.009 (100%)**	**DTDP-GSH, then NEM:**	**-0.077 ±0.020 (135 ±35%)**	**-0.006 ±0.001 (11 ±2%)**

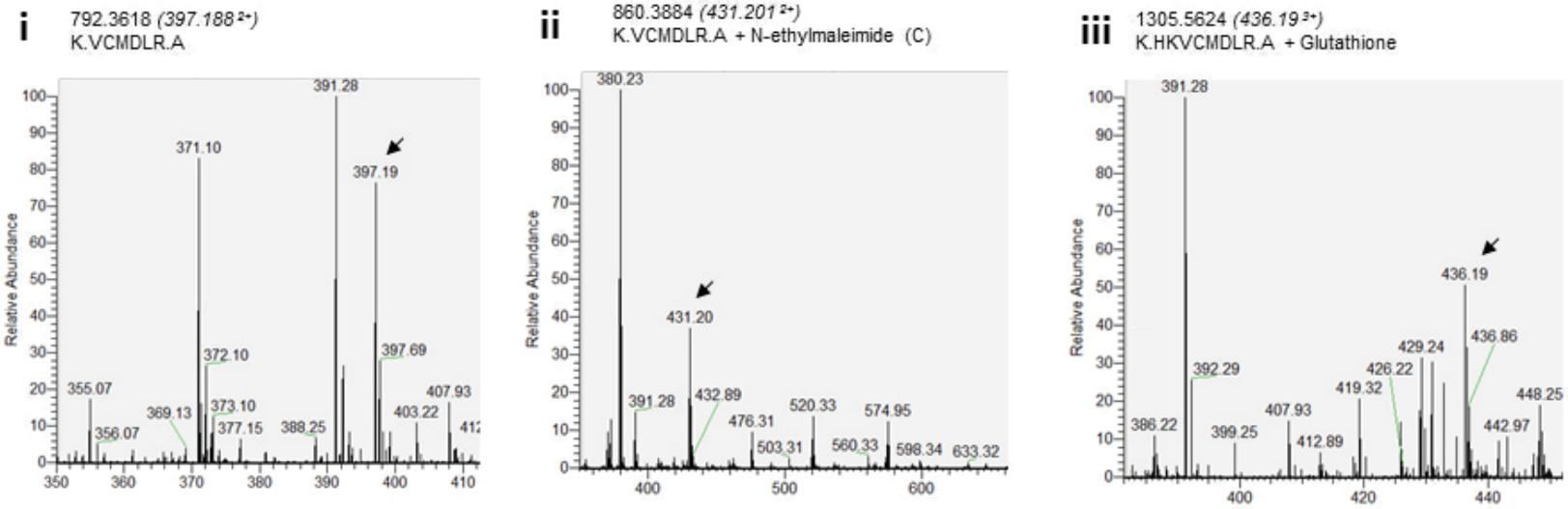
795
796 **Figure Legends**
797
798
799 Fig. 1. Separation of TnI_f and MLC1 for identification of S-glutathionylation. Western blots showing
800 streptavidin labelling of biotin-tagged glutathione, and subsequent reprobes for TnI and MLC1 in rat
801 EDL (type II) fibres treated with DTDP (1 min) and BioGEE, and then washed in high [KCl] to
802 dissociate myosin and associated myosin light chains (lanes labeled 'W') from the thin filaments and
803 structural proteins (lanes labeled 'F') (see Methods). Top panel shows corresponding actin signal on
804 12% Criterion Stain Free gel. The streptavidin signal corresponds to the TnI in the 'F' lanes, whereas
805 MLC1 is found in the wash ('W') lanes. The DTDP-BioGEE treatment increased Ca^{2+}-sensitivity in
806 the two EDL fibres shown by +0.192 and +0.177 pCa units.
807
808 Fig. 2. ESI-MS analysis (Orbitrap Elite) of TnI_f in EDL fiber samples for control (replicates R1 and
809 R2) and treated cases (S-glutathionylation with DTDP-GSH) (treated replicates R1 and R2). Top:
810 Identified tryptic peptide sequence of TnI_f fragments. The precursor refers to the intact peptide
811 sequence which dissociates to smaller fragment ions, following collision-induced dissociation. For
812 Tnl_f, ions of a particular mass-to-charge (m/z) ratio (precursor) were selected and fragment ions
813 were produced (products). Cysteine (Cys) residue indicated in right column. Bottom: Mass
814 spectrum for (i) VCMDLR (m/z 397.188^{2+}), (ii) VCMDLR + Nethylmaleimide (precursor m/z
815 431.201^{2+}) and (iii) HKVCMDLR + Glutathione (m/z 436.19^{3+}) generated from the Xcalibur
816 software platform. Charged m/z products are indicated.
817
818
819 Fig. 3. Ascorbate partially reverses the effect of SNAP on Ca^{2+} sensitivity of contractile apparatus.
820 *A*: Isometric force production in a skinned type II fibre from rat EDL muscle exposed to a sequence of
821 solutions with progressively higher free [Ca^{2+}] (pCa starting at >9, 6.49, 6.31, 6.11, 5.97, 5.84, 5.57,
822 4.7, then >9 again), before and after successive treatments with SNAP (2 mM, 2 min), ascorbate
823 (2 mM, 10 min) and DTT (10 mM, 10 min), all applied in pCa >9 solution. Horizontal arrows indicate
824 force level reached at pCa 5.84 in each force-pCa staircase. Lack of response to Sr^{2+} solution at pSr 5.2
825 confirmed fibre as type II. *B*: Hill curves for force staircases as numbered in A.
826

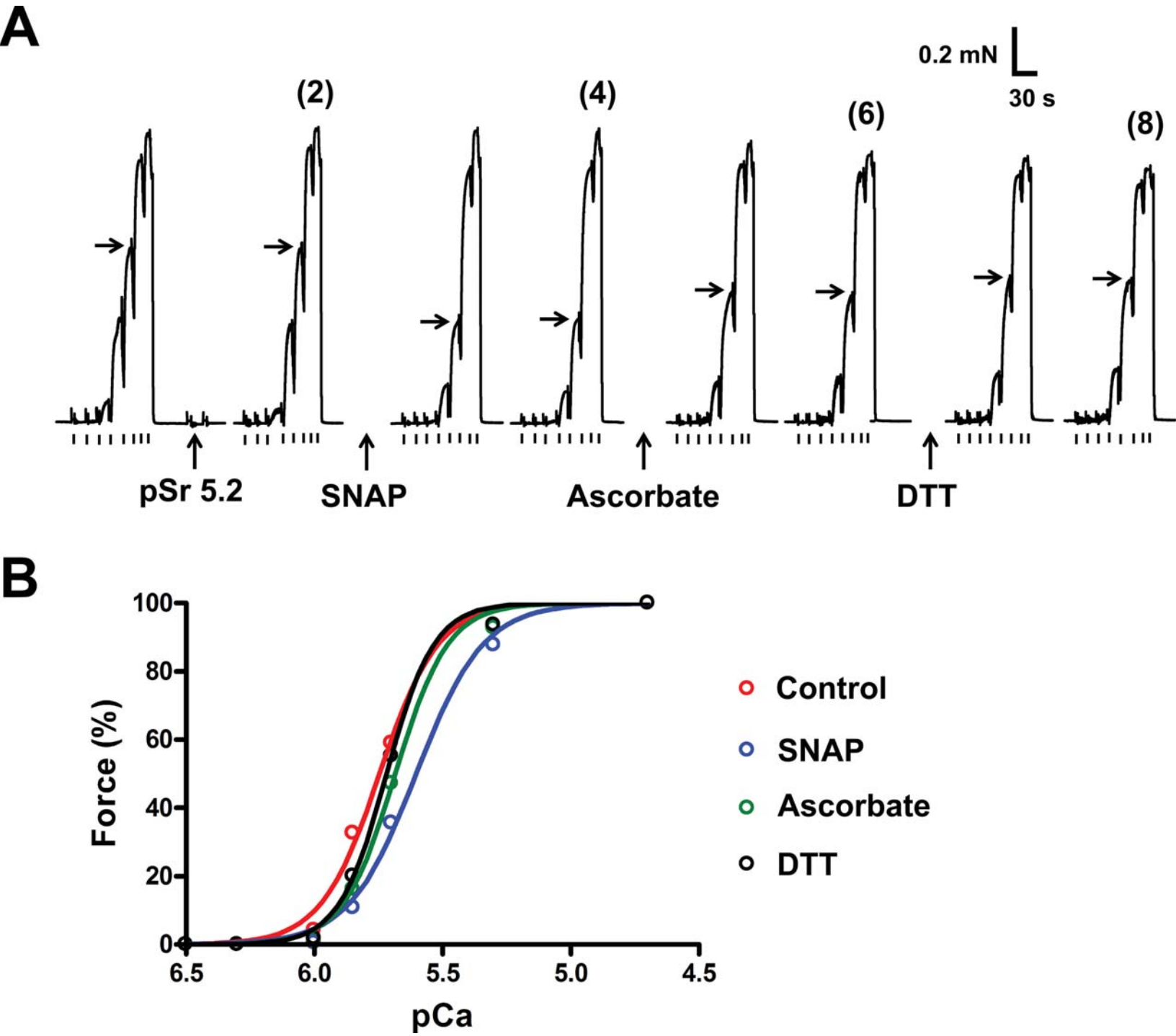
827 Fig. 4. S-glutathionylation treatment blocks effect of S-nitrosylation treatment on Ca^{2+} sensitivity.
828 *A*: force-pCa relationships in a rat EDL type II fibre before (control) and following each of the
829 indicated successive treatments (in order 1 to 6). Force-pCa relationship determined twice for each
830 case, displaying virtually identical behaviour; only second of each pair shown. Treatments: GSNO
831 (2 mM, 2 min); GSSG (10 mM, pH 8.5, 15 min); DTT (10 mM, 10 min); all applied at pCa >9. *B*:
832 pCa_{50} values for Hill fits in *A*, expressed relative to initial control level (CON). GSNO treatment
833 caused little or no change in pCa_{50} after the fibre had been pre-treated with GSSG. pCa_{50} values in
834 panel B adjusted to take into account the small progressive decline in pCa_{50} that occurs upon each
835 repeated force-pCa staircase irrespective of any treatment (see Methods).
836
837
838 Fig. 5. SNAP treatment prevents S-glutathionylation of TnI_f and also block by NEM. *A*: Western blot
839 with anti-GSH in samples composed of 5 skinned EDL fibre segments. Lane 1: fibres given only the
840 control treatment, 5 min wash in pCa >9 solution. Lane 2: initial wash and then standard DTDP-GSH
841 treatment. Lane 3: initial wash and then treatment with 10 mM SNAP for 10 min followed by standard
842 DTDP-GSH treatment. Lower panel: reprobe of membrane for TnI. 15% SDS gel. *B*: mean (+SE) of
843 TnI_f S-glutathionylation signal following indicated treatments, examined on 3 independent gels. GSH
844 signal for each sample on a gel first normalized by density of corresponding TnI_f signal, and then all
845 values expressed relative to DTDP-GSH sample run on same gel. * indicates significantly different
846 from DTDP-GSH treatment, and # indicates significantly different from SNAP/NEM/DTDP-GSH
847 treatment (rightmost case) (one way ANOVA with Newman-Keuls post-hoc analysis).
848
849 Fig. 6. S-glutathionylation and S-nitrosylation treatments each protect the other effect from block by
850 NEM. *A* & *B*: Hill fits to force-pCa data in a rat EDL type II fibre subjected to indicated sequence of
851 treatments (only treatments 6 to 11 shown for clarity), and relative pCa_{50} values for entire sequence
852 (treatments 1 to 11); GSNO (2 mM, 10 min), NEM (25 μM, 2 min), DTT (10 mM, 10 min), and
853 standard DTDP-GSH treatment. Pre-treatment with GSNO prevented NEM from blocking the effect of
854 the DTDP-GSH treatment. ***C***, Similar data from another EDL fibre showing GSSG pre-treatment
855 (10 mM in pH 8.5, 15 min) prevented NEM block of GSNO effect. Data in A and B adjusted to take
856 into account small decrease in Ca^{2+}-sensitivity occurring with each successive force-pCa staircase,
857 derived here from average shift in pCa_{50} for post-DTT responses (~ -0.007 pCa unit shift per staircase

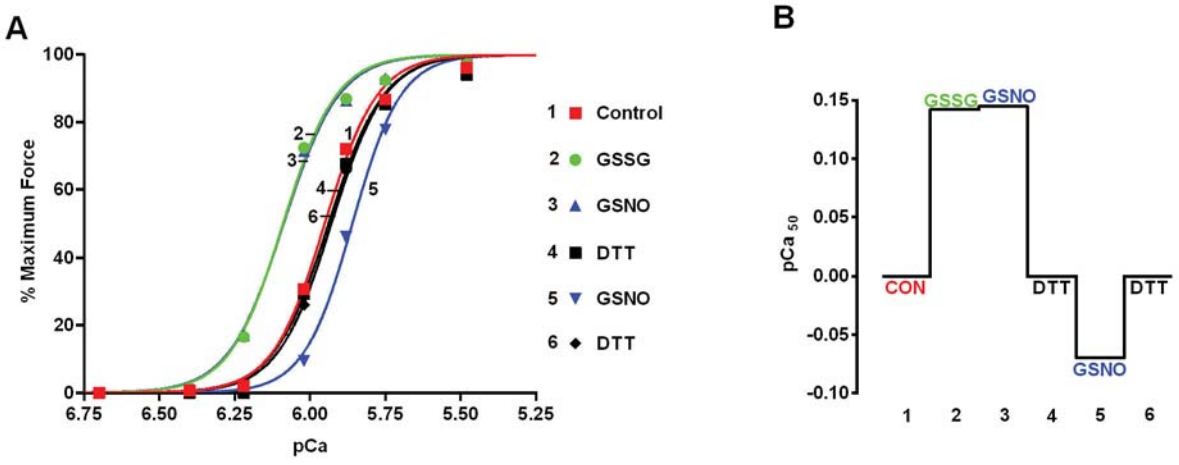
858 pair in both *A* and *B*). Two force-pCa staircases elicited after each treatment, giving very similar
859 responses; data shown only for second of each pair.
860
861 Fig. 7. SNAP treatment prevents NEM block of TnI_f labelling with sulphydryl-reactive biotin.
862 Lower panel: Streptavidin labelling of biotin-tagged proteins in single skinned EDL fibre segments
863 subjected to indicated treatments: SNAP (10 mM, 2 min); NEM (200 μM, 2 min), DTT (10 mM,
864 10 min), and HPDP-biotin (100 μM, 1 min). 12.5% SDS gel. Arrow indicates TnI. Top panel:
865 reprobe of membrane for TnI.
866
867 Fig. 8. Biotin-switch assay showing S-nitrosylation of TnI_f upon GSNO or SNAP treatment.
868 *A*: Streptavidin labelling of biotin tag on TnI_f, MLC1 and MLC3 in EDL fibres given GSNO (lanes 3 &
869 6) or SNAP treatment (lanes 2 & 5), and negative controls without such treatment (lanes 1 & 4) (see
870 Methods). *B*: Example where labelling of TnI_f and MLC1 overlay. S-nitrosylation of TnI_f/MLC1 with
871 GSNO treatment (lanes 5 & 9) was substantial compared to the maximal level of labelling observed in
872 the positive controls (lane 1: with no NEM block; lanes 2 & 3: GSNO treatment with reversal by DTT
873 instead of ascorbate), and decreased by S-glutathionylation pre-treatment with GSSG (lanes 6 & 10) or
874 DTDP-GSH (lanes 7 & 11). Fibre segments in this example were first treated with Triton-X-100 to
875 remove all membranes, and in this case biotin labelling of TnC was also observed, even in the negative
876 controls (lanes 4, 8 & 12), likely caused by dissociation of TnC from the troponin complex over the
877 course of the 12 hr exposure to biotin (see Methods). Middle panel: reprobe for TnI; band most readily
878 apparent in negative control cases, where there was no interference by initial strong streptavidin signal.
879 Three EDL fibre segments run in each lane. 4-15% Criterion Stain Free gels; actin band on Stain Free
880 gels shown in top panels of *A* & *B*.

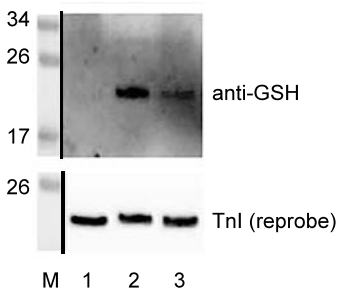

Stain Free gel
(actin)
170
130
95
72
55
43
34
25
17
streptavidin
probe
34
25
17
TnI (2nd probe)
34
25
17
MLC1 (3rd probe)
MWt
W F W F

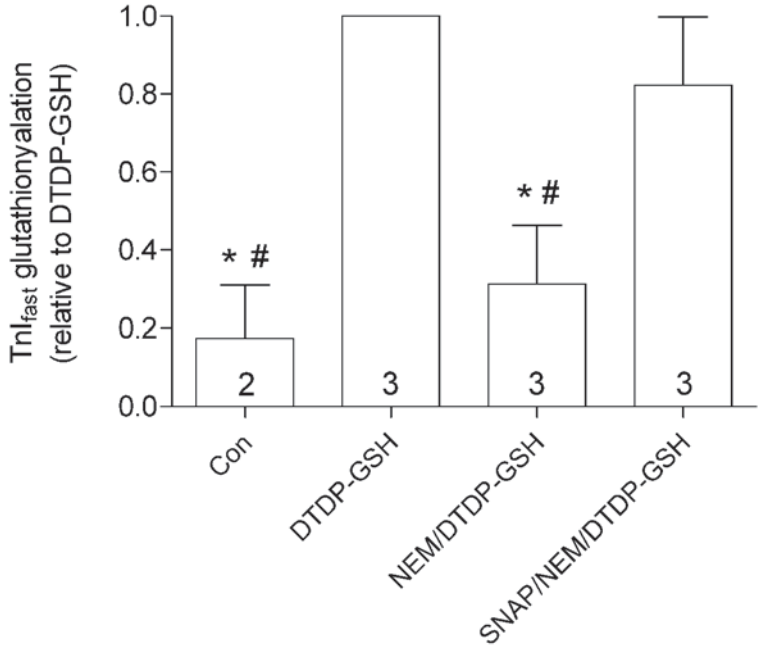
Sample (replicate)	Start-end of sequence	m/z experimental (precursor)	m/z calculated (precursor)	Tryptic fragment sequence	Cysteine residue
Control R1 Control R2	133-138	792.3618 792.3621	792.3622	VCMDLR	134
Control R1 Control R2	133-138	860.3884 860.3884	860.3884	VCMDLR + N-ethylmaleimide	134
Treat R1 Treat R2	131-138	1305.5624 1305.5629	1305.5628	HKVCMDLR + Glutathione	134

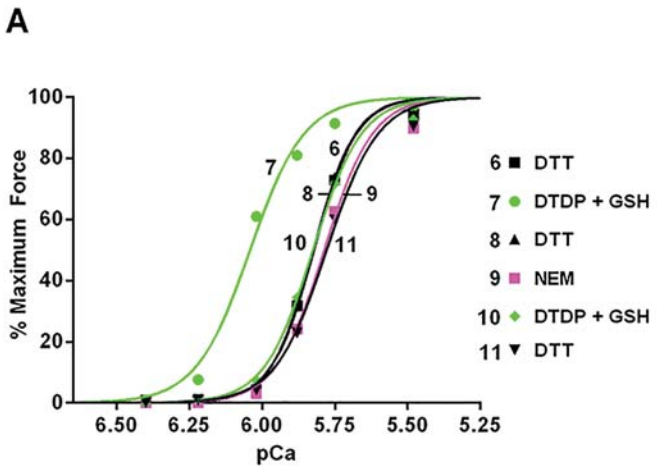


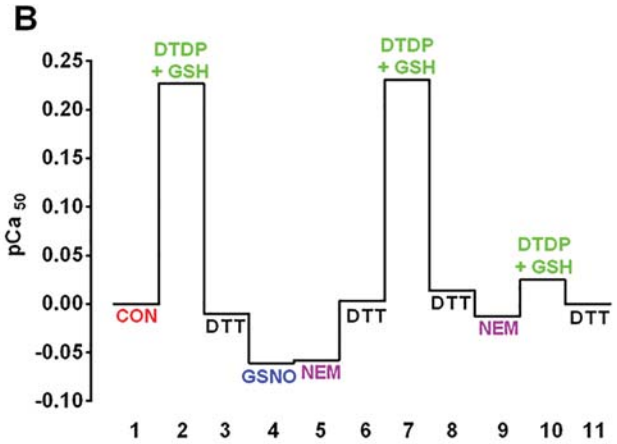

A
0.2 mN
30 s
(2)
(4)
(6)
(8)
pSr 5.2
SNAP
Ascorbate
DTT
B
Force (%)
100
80
60
40
20
0
6.5
6.0
5.5
5.0
4.5
pCa
Control
SNAP
Ascorbate
DTT

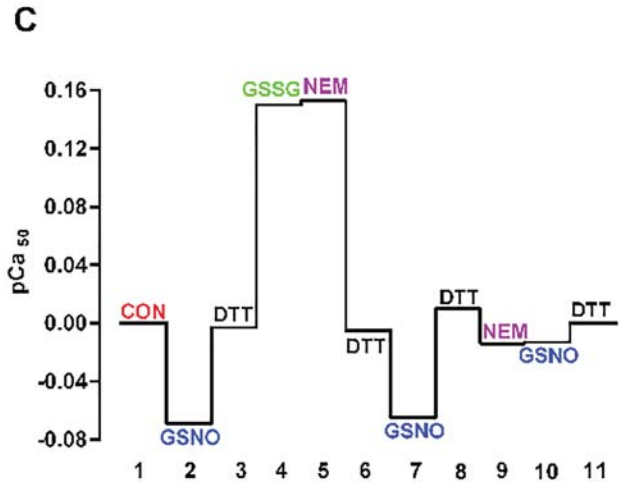

A
% Maximum Force
pCa
100
80
60
40
20
0
6.75
6.50
6.25
6.00
5.75
5.50
5.25
1 Control
2 GSSG
3 GSNO
4 DTT
5 GSNO
6 DTT
B
pCa 50
0.15
0.10
0.05
0.00
-0.05
-0.10
CON
GSSG
GSNO
DTT
GSNO
DTT
1
2
3
4
5
6

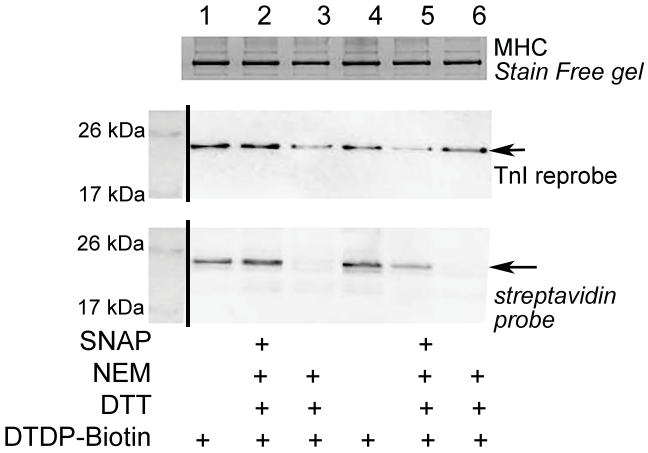

34
26
17
anti-GSH
26
TnI (reprobe)
M 1 2 3

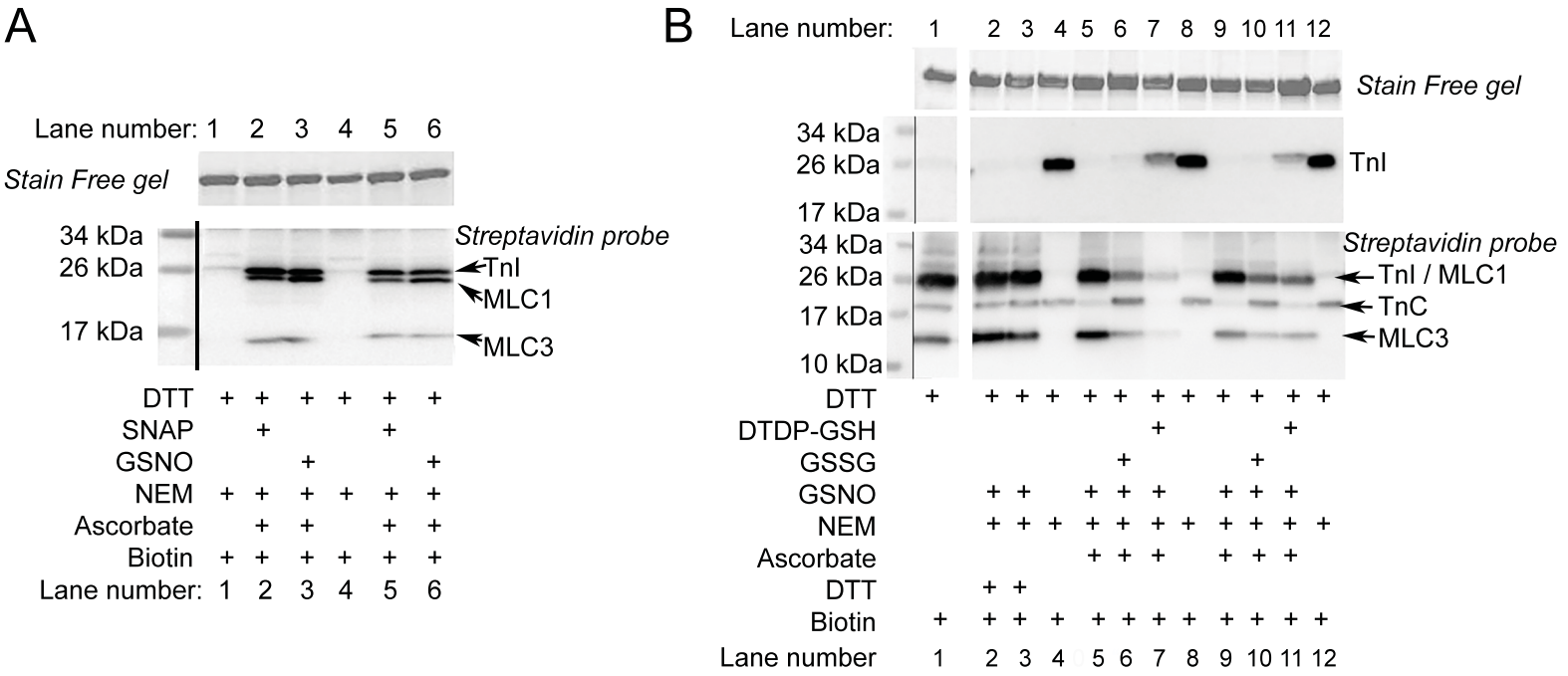

TnI$_{fast}$ glutathionyalation
(relative to DTDP-GSH)
1.0
0.8
0.6
0.4
0.2
0.0
* #
* #
2
3
3
3
Con
DTDP-GSH
NEM/DTDP-GSH
SNAP/NEM/DTDP-GSH


A
% Maximum Force
pCa
6 DTT
7 DTDP + GSH
8 DTT
9 NEM
10 DTDP + GSH
11 DTT


B
pCa 50
DTDP + GSH
DTDP + GSH
DTDP + GSH
CON
DTT
GSNO
NEM
DTT
DTT
NEM
DTT


C
pCa 50
GSSG
NEM
CON
GSNO
DTT
DTT
GSNO
DTT
NEM
GSNO
DTT


1 2 3 4 5 6
MHC
Stain Free gel
26 kDa
17 kDa
TnI reprobe
26 kDa
17 kDa
streptavidin
probe
SNAP + +
NEM + + + +
DTT + + + +
DTDP-Biotin + + + + + +


A
Lane number: 1 2 3 4 5 6
Stain Free gel
34 kDa
26 kDa
17 kDa
Streptavidin probe
TnI
MLC1
MLC3
DTT + + + + + +
SNAP + +
GSNO + +
NEM + + + + + +
Ascorbate + + + +
Biotin + + + + + +
Lane number: 1 2 3 4 5 6
B
Lane number: 1 2 3 4 5 6 7 8 9 10 11 12
Stain Free gel
34 kDa
26 kDa
17 kDa
TnI
34 kDa
26 kDa
17 kDa
10 kDa
Streptavidin probe
TnI / MLC1
TnC
MLC3
DTT + + + + + + + + + + + +
DTDP-GSH + +
GSSG + +
GSNO + + + + + + + + +
NEM + + + + + + + + + + +
Ascorbate + + + + + +
DTT + +
Biotin + + + + + + + + + + + +
Lane number 1 2 3 4 5 6 7 8 9 10 11 12