

**S-glutathionylation of Troponin I (fast) increases contractile apparatus  
Ca<sup>2+</sup>-sensitivity in fast-twitch muscle fibres of rats and humans**

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**Running Head:** S-glutathionylation of TnI<sub>f</sub> increases Ca<sup>2+</sup>-sensitivity

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## **Non-Technical Summary**

Reactive oxygen-based molecules generated within muscle fibres in both exercise and pathological conditions can greatly affect muscle function. These and consequent reactions can lead to either decreased or increased force response by the contractile proteins, but the mechanisms are unknown. This study demonstrates that the increase in force response appears to be due to a specific chemical process, known as S-glutathionylation, of a particular cysteine residue present on the troponin I molecule in fast-twitch muscle fibres, which is involved in sensing and responding to changes in intracellular calcium levels. S-glutathionylation can occur when glutathione, the primary cellular anti-oxidant, reacts with oxidized cysteines residues. S-glutathionylation of troponin I not only helps protect the molecule from oxidative stress, but evidently also makes the contractile apparatus much more sensitive to calcium ions. This process seemingly occurs in exercising humans and is likely to be an important mechanism helping delay onset of muscle fatigue.

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## Abstract

Oxidation can decrease or increase the  $\text{Ca}^{2+}$ -sensitivity of the contractile apparatus in rodent fast-twitch (type II) skeletal muscle fibres, but the reactions and molecular targets involved are unknown. This study examined whether increased  $\text{Ca}^{2+}$ -sensitivity is due to S-glutathionylation of particular cysteine residues. Skinned muscle fibres were directly activated in heavily-buffered  $\text{Ca}^{2+}$  solutions to assess contractile apparatus  $\text{Ca}^{2+}$ -sensitivity. Rat type II fibres were subjected to S-glutathionylation by successive treatments with 2,2'-dithiodipyridine (DTDP) and glutathione (GSH), and displayed a maximal increase in  $\text{pCa}_{50}$  ( $=-\log_{10} [\text{Ca}^{2+}]$  at half maximal force) of  $\sim 0.24$  pCa units, with little or no effect on maximum force or Hill coefficient. Partial similar effect was produced by exposure to oxidized glutathione (GSSG, 10 mM) for 10 min at pH 7.1, and near maximal effect by GSSG treatment at pH 8.5. None of these treatments significantly altered  $\text{Ca}^{2+}$ -sensitivity in rat type I fibres. Western blotting showed that both the DTDP-GSH and GSSG-pH8.5 treatments caused marked S-glutathionylation of the fast troponin I isoform ( $\text{TnI}_f$ ) present in type II fibres, but not of troponin C (TnC) or myosin light chain 2. Both the increased  $\text{Ca}^{2+}$ -sensitivity and glutathionylation of  $\text{TnI}_f$  were blocked by N-ethylmaleimide (NEM). S-Nitrosoglutathione (GSNO) also increased  $\text{Ca}^{2+}$ -sensitivity, but only in conditions where it caused S-glutathionylation of  $\text{TnI}_f$ . In human type II fibres from *vastus lateralis* muscle, DTDP-GSH treatment also caused similar increased  $\text{Ca}^{2+}$ -sensitivity and S-glutathionylation of  $\text{TnI}_f$ . When the slow isoform of TnI in type I fibres of rat was partially substituted ( $\sim 30\%$ ) with  $\text{TnI}_f$ , DTDP-GSH treatment caused a significant increase in  $\text{Ca}^{2+}$ -sensitivity ( $\sim 0.08$  pCa units).  $\text{TnI}_f$  in type II fibres from toad and chicken muscle lack Cys133 present in mammalian  $\text{TnI}_f$ , and such fibres showed no change in  $\text{Ca}^{2+}$ -sensitivity with DTDP-GSH nor any S-glutathionylation of  $\text{TnI}_f$  (latter examined only in toad). Following 40 min of cycling exercise in human subjects (at  $\sim 60\%$  peak oxygen consumption),  $\text{TnI}_f$  in *vastus lateralis* muscle displayed a marked increase in S-glutathionylation ( $\sim 4$  fold). These findings show that S-glutathionylation of  $\text{TnI}_f$ , most likely at Cys133, increases the  $\text{Ca}^{2+}$ -sensitivity of the contractile apparatus, and that this occurs in exercising humans, with likely beneficial effects on performance.

## Abbreviations List

CaEGTA, calcium bound to EGTA; CrP, creatine phosphate; DTDP, 2,2'-dithiodipyridine; EDL, extensor digitorum longus; GSH, reduced glutathione; GSNO, S-nitrosoglutathione; HDTA, hexamethylene-diamine-tetraacetate;  $h$ , Hill coefficient;  $H_2O_2$ , hydrogen peroxide;  $n$ , number of fibres; NEM, N-ethylmaleimide;  $pCa$ ,  $-\log_{10} [Ca^{2+}]$ ;  $pCa_{50}$ ,  $pCa$  producing half maximal force;  $pSr_{50}$ ,  $-\log_{10} [Sr^{2+}]$ ;  $pSr_{50}$ ,  $pSr$  producing half maximal force; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetyl-penicillamine; TFP, trifluoperazine; TnC, troponin C; TnI, troponin I; TnT, troponin T;  $V_{O_2,peak}$ , peak oxygen consumption.

## Introduction

Reactive oxygen and nitrogen species are generated in skeletal muscle with normal activity and also in pathological conditions, and affect many aspects of muscle function both in the short and long term (Smith & Reid, 2006; Supinski & Callahan, 2007; Allen *et al.*, 2008; Powers & Jackson, 2008; Lamb & Westerblad, 2011). Application of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) to intact fast-twitch muscle fibres of the mouse was found to initially cause an increase in force production by increasing the  $\text{Ca}^{2+}$ -sensitivity of the contractile apparatus, with prolonged exposure leading to a subsequent net decrease in  $\text{Ca}^{2+}$ -sensitivity (Andrade *et al.*, 1998; Andrade *et al.*, 2001). These effects were fully reversible by application of the reducing agent, dithiothreitol (DTT), suggestive that the changes involved reversible oxidation of cysteine residues. Using skinned muscle fibres we have previously shown that the increase in  $\text{Ca}^{2+}$ -sensitivity is evidently not due to a direct effect of  $\text{H}_2\text{O}_2$  on the contractile apparatus, and instead possibly results from the  $\text{H}_2\text{O}_2$  interacting with myoglobin and reduced glutathione (GSH) within in the fibre (Lamb & Posterino, 2003; Murphy *et al.*, 2008), resulting in S-glutathionylation of unknown cysteine residues on the contractile proteins (i.e.  $\text{RSH} \rightarrow \text{RSSG}$ ). This could come about by generation in the cytoplasm of the reactive thiyl radical ( $\text{GS}^\bullet$ ) and/or S-nitrosoglutathione (GSNO) (Dutka *et al.*, 2011b), or simply by the GSH present reacting with oxidized cysteine residues on the contractile proteins (see (Lamb & Westerblad, 2011)). S-glutathionylation is now recognized as an important mechanism that not only helps prevent disruption of protein function by oxidative stress, but also can itself regulate protein function (Klatt & Lamas, 2000; Dalle-Donne *et al.*, 2007).

In our previous studies, the presumed S-glutathionylation of the contractile apparatus was mostly elicited by treating the skinned fibre with the reactive disulphide, 2,2'-dithiodipyridine (DTDP) to oxidize cysteine residues and then exposing the fibre to GSH for a relatively short period (Lamb & Posterino, 2003). **The resulting large increase in  $\text{Ca}^{2+}$ -sensitivity only occurred in fast-twitch (type II) fibres, and was reversed by DTT or relatively prolonged exposure to GSH.** The DTDP treatment

itself causes a moderate decrease in  $\text{Ca}^{2+}$ -sensitivity (decrease in  $\text{pCa}_{50}$  of  $\sim 0.065$  pCa units), and the brief subsequent GSH exposure causes a very large increase in sensitivity (by  $\sim 0.28$  pCa units), resulting in a net increase by  $\sim 0.23$  pCa units, which is no different from the outcome if the two treatments are applied in immediate succession without examining the shift occurring with the DTDP treatment alone. Following reversal by DTT, a repeat of the DTDP-GSH treatment elicits virtually identical results. Similar DTDP-GSH treatment in type I (slow-twitch) rat fibres caused no increase in  $\text{Ca}^{2+}$ -sensitivity whatsoever (Lamb & Posterino, 2003).

The aim of the present study was to identify the protein(s) in type II fibres responsible for the increased  $\text{Ca}^{2+}$ -sensitivity to DTDP-GSH treatment, verifying by western blotting that this indeed involves S-glutathionylation, and if possible also identifying the specific cysteine residues involved. The experiments were performed predominantly using skinned muscle fibres, with the first goal being to determine whether treatment with oxidized glutathione (GSSG) produced effects similar to DTDP-GSH treatment, which would verify that the effects of the latter were not the result of some unintended action of DTDP. As a low concentration of the alkylating agent, N-ethylmaleimide (NEM), has been shown to specifically block the increase in  $\text{Ca}^{2+}$ -sensitivity to applied  $\text{H}_2\text{O}_2$  in intact fibres (Andrade *et al.*, 2001), we examined the ability of NEM to block both the increased  $\text{Ca}^{2+}$ -sensitivity and S-glutathionylation of specific proteins. As the fast isoform of troponin I ( $\text{TnI}_f$ ) was found to undergo S-glutathionylation in tight accord with the  $\text{Ca}^{2+}$ -sensitivity increase, we examined whether substituting  $\text{TnI}_f$  into type I fibres altered their response to DTDP-GSH treatment. We also examined whether the DTDP-GSH treatment affected type II fibres from toad and chicken muscle, as the  $\text{TnI}_f$  present in such fibres (Wilkinson & Grand, 1978) lacks one particular highly-reactive and accessible cysteine residue present in mammalian  $\text{TnI}_f$ , Cys133 (Chong & Hodges, 1982; Tao *et al.*, 1990; Park *et al.*, 1994). Finally, we examined the effect of DTDP-GSH treatment in type I and type II fibres from human *vastus lateralis* muscle and whether the glutathionylation state of  $\text{TnI}_f$  was affected in exercise.

## Methods

### Muscle fibres and samples and ethical approval

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 (42 in total,  $\geq 5$  months old) 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*) that had been maintained at 15°C to lower their activity, were stunned and then killed by pithing, and the *iliofibularis* muscle removed. Two chickens were killed by overdose of intravenous phenobarbitone and a segment of pectoralis major muscle removed.

All protocols and procedures performed with human subjects were approved by the appropriate Human Research Ethics Committees at Victoria University, University of Melbourne and La Trobe University. Informed consent was obtained in writing from all subjects and the studies conformed to the standards set by the Declaration of Helsinki. The experiments on the effects of DTDP-GSH treatment on human skinned fibres were performed on fibres obtained by muscle biopsy in **three subjects (two males and one female, 19 – 27 years old, all healthy and recreationally fit)**. 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 (McKenna et al., 2006). An experienced medical practitioner took all biopsies at approximately constant depth. The excised muscle sample was rapidly blotted on filter paper to remove excess blood and placed in paraffin oil (Ajax Chemicals, Sydney, Australia) at 10°C for 45 min before individual muscle fibers were dissected. The effect of exercise on S-glutathionylation in muscle of human subjects was performed using tissue available from a previously published study (Merry et al., 2010). Briefly, tissue samples were obtained from 5 healthy, recreationally active male subjects (age  $23 \pm 2$  years) participating in a double-blind

randomised cross-over design exercise study with counterbalanced testing order. A *vastus lateralis* muscle biopsy was obtained as above with the subjects rested, and also on a different date at least 2 weeks apart after the subjects had completed 40 min of cycling exercise at ~60% of peak oxygen consumption ( $V_{O_{2,peak}}$ ) (see (Merry et al., 2010)). Muscle samples were frozen in liquid nitrogen while still in the biopsy needle within 6–12 s following the cessation of exercise. Muscle samples were stored in liquid nitrogen for later analysis and were obtained from the contralateral leg during the second trial.

### **Preparations and force recording**

Rat and toad whole muscles, and muscle biopsies from human and chicken muscle, were pinned at approximately resting length under paraffin oil (Ajax Chemicals, Sydney, Australia) in a petri dish, and kept cool (~10°C) on an icepack. Individual fibre segments were mechanically-skinned with jeweller's forceps and then mounted at 120 % of resting length on a force transducer (AME801, SensoNor, Horten, Norway) with a resonance frequency >2 kHz. The skinned fibre segment was then equilibrated for > 2 min in a perspex bath containing 2 ml of relaxing solution (see below). Force responses were recorded using a Bioamp pod and Powerlab 4/20 series hardware (ADInstruments, Sydney, Australia). All experiments were performed at room temperature (~23 ±2 °C).

### **Skinned fibre solutions**

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. As previously described (Lamb & Posterino, 2003), the 'relaxing' solution contained (in mM): **EGTA**, 50; total ATP, 8; creatine phosphate (CrP), 10; Na<sup>+</sup>, 36; K<sup>+</sup>, 126; total Mg<sup>2+</sup>, 8.5; total; HEPES, 90; pH 7.1 and pCa >9. The maximum Ca<sup>2+</sup>-activating solution 'max' contained 50 mM CaEGTA and had a pCa ~4.7, with total Mg<sup>2+</sup> adjusted to maintain 1 mM free (see (Stephenson & Williams, 1981) for apparent affinity constants). These two solutions were mixed in appropriate



ratio to produce solutions with pCa in the range 6.7 to 4.7. All solutions had an osmolality of  $295 \pm 5$  mosmol  $\text{kg}^{-1}$ . Similar strontium-based solutions (with pSr 5.2 =  $-\log_{10}[\text{Sr}^{2+}]$  in range >9 to 4.0) were made by mixing relaxing solution with a Sr-EGTA solution similar to the maximum  $\text{Ca}^{2+}$ -activating solution. Exposure to a solution at pSr 5.2 was used to ascertain the predominant troponin C (TnC) isoform present (see Results, and (O'Connell *et al.*, 2004; Trinh & Lamb, 2006)). Subsequent western blotting was used to confirm the TnC isoform present in all human, chicken, toad and some rat fibres.

A 100 mM stock of reduced glutathione (GSH) was made in a potassium HDTA (hexa-methylene-diamine-tetraacetate) solution similar to the relaxing solution but with all EGTA replaced with HDTA; the pH of the stock was re-adjusted to 7.10 with KOH, and then diluted 20-fold to give 5 mM in the final solution. A 100 mM stock solution of 2, 2'-dithiodipyridine (DTDP) was made in absolute ethanol and diluted 1000-fold in the final solution to 100  $\mu\text{M}$ ; matching control solutions with the same amount of ethanol (0.1 %) had no noticeably different effect than controls without ethanol. Similarly, NEM was made as a 200 mM or 25 mM stock in ethanol and diluted 1000-fold in the final solution. DTT was added to relaxing solution at 10 mM final concentration from a 1 M stock made in double distilled water. S-nitrosoglutathione (GSNO) can cause either S-nitrosylation or S-glutathionylation of a protein thiol. GSNO was dissolved in solution and either applied immediately to the fibre (i.e. within 30 sec, termed 'GSNO<sub>imm</sub>') or applied ~10 min later (i.e. a 10 min delay, termed 'GSNO<sub>del</sub>'), always with an exposure period of 2 min; it was found that the effects of the GSNO treatment was quite different in the two cases. SNAP (S-nitroso-N-acetylpenicillamine, 5 mM) was applied immediately after addition to solution (i.e. within 30 sec).

Except where stated, fibres were never activated in the presence of any of the treatments and were simply exposed to each treatment, washed in standard solution/relaxing solution as appropriate, and then transferred back into the solutions in which the force responses were elicited.

### **Contractile apparatus experiments and analysis**

The force-Ca<sup>2+</sup> relationship was determined in each fibre as previously described (Lamb & Posterino, 2003; Murphy *et al.*, 2008) by exposing the skinned fibre segment to a sequence of solutions heavily-buffered at progressively higher free [Ca<sup>2+</sup>] (pCa >9 to 4.7, the latter eliciting maximum force), and then the fibre 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<sup>2+</sup>] 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 fibre segment, using GraphPad Prism 4 software, yielding separate pCa<sub>50</sub> value and *h* values (pCa at half-maximum force and Hill coefficient, respectively) for every case. Maximum force reached in each force-[Ca<sup>2+</sup>] sequence was expressed relative to the control level before any treatment in the given fibre, after correcting for the small decline occurring with each repetition of the force staircase (typically ~2 to 3% in EDL fibres), as gauged from the initial control repetitions in the given fibre (see also (Murphy *et al.*, 2008)).

### **Western blotting**

Non-reducing SDS-PAGE was used for the determination of S-glutathionylation and biotin labeling in rat, human and toad fibres, whereas reducing SDS-PAGE was used for the other analyses of rat fibres following troponin exchange experiments and other experiments with human, chicken and toad fibres. The S-glutathionylation experiments on rat and toad fibres was based on our previously described small-sample methodology (Murphy *et al.*, 2009; Dutka *et al.*, 2011a) with each sample consisting of 4 or 5 skinned fibre segments (~60 µg wet wt). Western blotting of human *vastus lateralis* muscle fibres was performed using the individual skinned fibre segments in which force measurements had been made (e.g. Fig. 8). In all cases the entire fibre constituents were examined

without discarding any fraction. Where multiple skinned fibre segments were examined for S-glutathionylation, they were tied together with a silk suture, washed in relaxing solution for at least 5 min and then transferred successively to the various treatment solutions as required. For biotin-labeling experiments, following an initial 5 min wash in relaxing solution, individual mechanically-skinned EDL fibre segments were treated for either 1 or 5 min with 100  $\mu$ M EZ-Link Biotin-HPDP (ThermoScientific). Treatment with EZ-Link-Biotin-HPDP results in the formation of a disulphide bond between free sulfhydryl (-SH) groups in the fibres and the reagent, resulting in a biotin tag on any reacted proteins. As the last step for all of the above, fibre samples were placed in relaxing solution with 5 mM NEM for 5 min to block free sulfhydryl sites and then placed in non-reducing buffer for SDS-PAGE (final concentration: 125 mM Tris pH 6.8, 10% glycerol, 4% SDS, 0.01% bromophenol blue, 5 mM NEM). With the troponin exchange experiments (see below) (e.g. Fig. 7), the individual soleus skinned fibre segments were mounted on the force transducer and force-pCa measurements made before and after the troponin exchange procedure, and then the fibre was collected into reducing SDS-PAGE buffer, which was similar to the non-reducing buffer described above except the NEM was replaced with the reducing agents urea (4 M) and mercaptoethanol (10%). Total proteins in fibres collected into either buffer was separated on various SDS-PAGE gels, (details provided in relevant figure legends, Criterion gels were from BioRad, Hercules, CA, USA) and then wet transferred to nitrocellulose for 60 min at 100 V in a circulating ice-cooled bath with transfer buffer containing 140 mM glycine, 37 mM Tris-base, and 20% methanol. Membranes were then variously probed with anti-GSH (mouse monoclonal, 1 in 1000, Cat No. 101-A, Virogen, Cincinnati, OH, USA), anti-TnI (rabbit polyclonal, 1 in 1000, Cat No. 4002, Cell Signaling Technology, Danvers, MA, USA), TnC (rabbit polyclonal, 1 in 400, Cat No. sc-20642, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-myosin light chain 2 (rabbit polyclonal, 1 in 200, Cat No. sc-15370, Santa Cruz Biotechnology), anti-actin (rabbit affinity isolated, Cat No. A2066, Sigma) all diluted in 1% bovine serum albumin in phosphate-buffered saline with 0.025% Tween. Following exposure to relevant secondary antibodies (or in the case of

biotin labeling, to streptavidin (1 in 20,000 in 1%BSA)) and a series of washes in Tris-buffered saline with Tween, chemiluminescent substrate (SuperSignal West Femto, Pierce) was applied to membranes and Western blot images taken with ChemiDoc XRS fitted with a charge-coupled device (CCD) camera using Quantity One software (Bio-Rad). With the membrane position unchanged, the white light source was switched on in order to obtain an image of the prestained molecular weight markers on the membrane.

In order to determine the level of S-glutathionylation in whole muscle before and after exercise in humans, two to four 10 µm cryosections were cut from each biopsy (Leica 1500 CM1950) and placed into microfuge tubes pre-equilibrated to -20°C (number of slices varied to keep total amount of tissue approximately the same in all cases). Cold physiological-based solution with 5 mM NEM added was added (10 µl) and the samples kept on ice for 2 min, before non-reducing buffer (see above) was added. Samples were stored at -20°C until analyzed by western blotting, as described above. To confirm that the S-glutathionylation effect could be induced in human muscle and measured biochemically, 10 µm sections were cut with the cryostat and placed onto a microscope slide. A circle was drawn around the section with a water repelling ink (Dako, Glostrup, Denmark) and the section was then treated with solutions as described for the rat fibres. Solutions were aspirated from the sections prior to the addition of the next solution. Following all treatment steps, the section was collected from the slide in the non-reducing buffer by sucking up into a pipette and kept at -20°C until western blotting.

### **Troponin extraction and exchange.**

All skinned fibres used in the troponin extraction and exchange experiments were first mounted on the transducer and then treated with Triton-X100 in relaxing solution (1% vol/vol) for 10 min (and then washed in relaxing solution) in order to remove membranous structures and possibly aid exchange rates. Troponin C extraction was achieved by bathing the skinned fibre in a K-EGTA

rigor solution with 68 mM EGTA, 5 mM EDTA and 90 mM Hepes (pH to 7.1 with KOH) and no added  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , plus 0.5 mM trifluoperazine (TFP) (adapted from (Morris *et al.*, 2001)). The fibre was repetitively cycled for 6 min periods between the TFP-rigor solution and relaxing solution, for a total exposure to the TFP-rigor solution of 30 min. This was sufficient to abolish >99% of active force to the maximal  $\text{Ca}^{2+}$ -activating solution in type II fibres. Extraction of both troponin C and troponin I was achieved by bathing the skinned fibre in relaxing solution with 10 mM vandate for 10 min (see (Kogler *et al.*, 1998)). Following troponin extraction, the skinned fibre was bathed for 15 min in relaxing solution with 2 mM DTT and fast troponin either from porcine muscle (Sigma, T2275), or rabbit muscle (Ocean Biologicals Inc, Seattle, WA, USA), the latter applied as TnC alone, or equal part mixtures of TnI and TnT, or TnC, TnI and TnT (3 to 10 mg/ml). Troponin exchange (without initial troponin extraction) was achieved by bathing the skinned fibre segment for 1 hr in a low ionic strength rigor solution with zero  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (mM: EGTA, 2.5; EDTA, 2.5; Hepes, 10, pH 7.1 with KOH) with porcine or rabbit fast troponin (10 mg/ml) and 2 mM DTT.

### Statistics

Values are presented as mean  $\pm$  standard error of the mean (s.e.m.) (or mean  $\pm$  s.d., the sample standard deviation, where  $n = 2$ ), with  $n$  denoting the number of fibres examined. Statistical significance ( $P < 0.05$ ) was determined with Student's two-tailed paired  $t$ -test, or where values were not normally distributed by the Wilcoxin signed rank test. Data where  $n=2$  were not examined statistically.

## Results

### Effects of treatment with DTDP-GSH or GSSG

In agreement with our previous findings (Lamb & Posterino, 2003), when a skinned type II fibre from rat EDL muscle was treated sequentially with DTDP (100  $\mu$ M) for 5 min and then GSH (5 mM) for 2 min (denoted as the ‘standard’ DTDP-GSH treatment, all applied in relaxing solution,  $pCa > 9$ ), the  $Ca^{2+}$ -sensitivity of the contractile apparatus was greatly increased, as seen with the final treatment in Figure 1A. In the 18 EDL fibres examined, the  $pCa_{50}$  was increased on average by  $\sim 0.24$  pCa units (row 1 in Table 1), with only a slight decrease in the Hill coefficient ( $h$ ) ( $-11 \pm 4\%$ , from  $\sim 5.5$  to 5.0) and no detectable change in maximum  $Ca^{2+}$ -activated force. The effects of the DTDP-GSH treatment were fully reversed with the reducing agent, DTT (Table 1). The DTDP-GSH treatment had the exactly same effects in fibres treated with Triton-X100 (not shown), indicating that it acted directly on the contractile apparatus. Type II fibres obtained from rat soleus muscle, which are almost exclusively type IIA fibres (Bortolotto *et al.*, 2000; O’Connell *et al.*, 2004), showed exactly the same effects to DTDP-GSH as the EDL type II fibres (not shown).

The effect of the DTDP-GSH treatment was not noticeably different with DTDP (100  $\mu$ M) exposures of between 30 s and 5 min, and with GSH (5 mM) exposures of between 30 s and 2 min (all applied at  $pCa > 9$  without fibre activation). If the exposure time to DTDP was made even shorter, the increase in  $pCa_{50}$  was smaller, with a 5 s exposure causing only  $\sim 38$  and 43% of the maximal shift, and a 10 s exposure causing  $\sim 51$  and 57%, in the 4 EDL fibres examined.  $Sr^{2+}$  ions activate the TnC isoform present in fast-twitch fibres, but only at  $\sim 20$  fold higher concentration than for  $Ca^{2+}$  (Lynch *et al.*, 1995; O’Connell *et al.*, 2004; Trinh & Lamb, 2006), and it was found that the DTDP-GSH treatment increased the  $Sr^{2+}$ -sensitivity to a similar extent as the  $Ca^{2+}$ -sensitivity (maximal increase in  $pSr_{50}$   $+0.280 \pm 0.013$ , and in  $pCa_{50}$   $+0.241 \pm 0.012$  in above 4 fibres, and with similar proportional shift in  $pSr_{50}$  with the shorter DTDP exposures). At much higher concentration (e.g. 1 mM) DTDP treatment results in reduction in maximal force and  $Ca^{2+}$ -sensitivity in both type

II and type I fibres (Lamb & Posterino, 2003), probably by oxidizing cysteine residues on additional types of proteins.

In order to examine whether the effects of the standard DTDP-GSH treatment were attributable to S-glutathionylation, and not dependent on some additional effect of the initial DTDP oxidizing step, we compared the effects of oxidized glutathione (GSSG) as an alternative method of producing S-glutathionylation. We previously found that a 5 min exposure to 2.5 mM GSSG produced no significant change in  $\text{Ca}^{2+}$ -sensitivity (average  $+0.004$  pCa units in two EDL fibres) (Lamb & Posterino, 2003), and consequently here examined the effects a longer exposure at higher concentration. In the 3 EDL fibres examined, a 10 min exposure to 10 mM GSSG at pH 7.1 increased  $\text{pCa}_{50}$  by a mean of  $+0.055 \pm 0.010$ , which is  $\sim 25\%$  of the maximal DTDP-GSH effect. The ability of GSSG to S-glutathionylate a protein cysteine (RSH) is considerably enhanced if the latter is in its thiolate anion form ( $\text{RS}^-$ ) (Klatt & Lamas, 2000; Dalle-Donne *et al.*, 2007), which is favoured by increasing the solution pH, and so we next tested the effect of applying GSSG at pH 8.5 for 10 min. As such exposure at pH 8.5 itself causes a 'once-off' reduction in  $\text{Ca}^{2+}$ -sensitivity and maximum force in rat soleus fibres (Dutka *et al.*, 2011a), each EDL fibre was first subjected to the pH 8.5 conditions without GSSG, which resulted in reductions in  $\text{pCa}_{50}$  (by  $-0.079 \pm 0.009$  pCa units),  $h$  (by  $34 \pm 5\%$ ,  $n=12$ ), and maximum force (by  $6 \pm 1\%$ ) (e.g. see Fig. 1). A repeated exposure to pH 8.5 conditions had virtually no further effect (not shown). However, as seen in Figure 1, when 10 mM GSSG was present in the pH 8.5 solution, there was a very marked increase in the  $\text{Ca}^{2+}$ -sensitivity (mean increase in  $\text{pCa}_{50} \sim +0.19$ , row 4 in Table 1), with little or no change in either  $h$  or maximum force. This increase in  $\text{Ca}^{2+}$ -sensitivity was fully reversed by the standard DTT treatment (10 mM, 10 min), returning the level to approximately that present after the initial pH 8.5 exposure (Table 1).

When the standard DTDP-GSH treatment was applied after the 10 min GSSG-pH8.5 treatment, it resulted in little further increase in  $\text{Ca}^{2+}$ -sensitivity, but if the sensitivity increase was first reversed with DTT, DTDP-GSH treatment caused its usual large effect (see Fig 1). If however the DTDP-GSH treatment was applied following a submaximal GSSG treatment (i.e. 10 mM GSSG at pH 7.1, or 2.5 or 5 mM GSSG at pH 8.5), it caused a further increase in  $\text{Ca}^{2+}$ -sensitivity, but the net effect of the two sequential treatments (total shift of  $\text{pCa}_{50} \sim 0.23$ ) was still just the same as occurred with the DTDP-GSH treatment alone (Figure 2). All effects were fully reversed by DTT. These data strongly suggest that the DTDP-GSH and GSSG treatments increase  $\text{Ca}^{2+}$ -sensitivity by the same mechanism, that presumably being by S-glutathionylation.

### Effects of treatments in type I fibres

Skinned fibres obtained from rat soleus muscle were designated as type I or type II according to their response to  $\text{Sr}^{2+}$  (see Methods); type I fibres contain almost exclusively only the slow isoforms of troponin (e.g. see Fig. 7 later) and are relatively sensitive to activation by  $\text{Sr}^{2+}$ , whereas the type II fibres contain fast troponin isoforms and are relatively insensitive to  $\text{Sr}^{2+}$  (Lynch *et al.*, 1995; O'Connell *et al.*, 2004). In contrast to all rat type II fibres examined, rat type I fibres showed no significant change in sensitivity with either the DTDP-GSH treatment or the GSSG-pH 8.5 treatment (rows 6 & 7 in Table 1). Table 1 shows the changes in  $\text{pCa}_{50}$  occurring with the various treatments, as well as the effect of subsequent DTT treatment. **It should be borne in mind that subjecting** skinned fibres to repeated force-pCa measurements itself results in a small progressive decrease in  $\text{Ca}^{2+}$ -sensitivity (and maximum force) even without any treatment, with the shift being on average  $\sim -0.012$  pCa units per pair of force-pCa 'staircases' (see also (Lamb & Posterino, 2003; Murphy *et al.*, 2008)). **It is apparent that the DTDP-GSH and GSSG-pH 8.5 treatments had no detectable effect on  $\text{pCa}_{50}$  in rat type I fibres.**



### Effect of DTDP-GSH treatment during activation in type II fibres

All of the above treatments were applied with the fibres in the relaxed state (at  $pCa > 9$ ), and it was important to also consider whether the effect was any different in an activated fibre. Applying DTDP to type II fibres during submaximal activation affects  $Ca^{2+}$ -sensitivity (*increase* in  $pCa_{50}$  by  $\sim 0.05$  pCa units, and decrease in  $h$ ) in a different manner to that when applying the DTDP with the fibre relaxed (*decrease* in  $pCa_{50}$  by  $\sim 0.065$  pCa units) (Lamb & Posterino, 2003), possibly because the activation allows access to additional cysteine residues or interaction between relocated proteins. This activation-dependent effect appeared to be independent of the action of DTDP-GSH (Lamb & Posterino, 2003), but the combination of actions potentially complicates interpretation.

**Importantly**, it was found here that when DTDP was applied (for 15 s) whilst the fibre was *maximally activated* (in  $pCa$  4.7), it had effects similar to those in a resting fibre, decreasing  $pCa_{50}$  by  $\sim 0.08$  pCa units. This made it possible to readily compare the effects of DTDP-GSH treatment in the rested and maximally-activated states. DTDP was applied for only 15 s and then GSH for only 30 s in order to minimize the time the fibre was maintained in the maximally-activated state (which leads to irreversible decrement in force if very prolonged). It was found that such DTDP-GSH treatment produced virtually the same increase in  $Ca^{2+}$ -sensitivity irrespective of whether it was applied with the fibre relaxed ( $+0.206 \pm 0.020$  pCa units) or maximally activated ( $+0.197 \pm 0.018$  pCa units) (both treatments examined in same three type II EDL fibres).

### Block by NEM

Andrade et al (2001) found in intact fast-twitch fibres that a 3 min exposure to 25  $\mu M$  of NEM blocked the increase in contractile sensitivity that otherwise occurs with  $H_2O_2$  application, without the reductions in maximal force that occur with higher  $[NEM]$ , or longer exposure (Perkins *et al.*, 1997). It was found here that exposing type II EDL fibres for 2 min to 25  $\mu M$  NEM (at  $pCa > 9$ ) blocked  $>85\%$  of the increase in  $Ca^{2+}$ -sensitivity occurring with the DTDP-GSH and GSSG-pH 8.5 treatments (Table 1, rows 2 & 5) (e.g. Figure 3). The NEM treatment itself caused only a minor

decrease in maximum force ( $-2.3\% \pm 0.7\%$ ) and  $\text{Ca}^{2+}$ -sensitivity ( $\Delta\text{pCa}_{50} -0.047 \pm 0.008$  pCa units) ( $n=7$ ). As expected, none of the effects of the alkylating agent NEM were reversed by DTT.

### Western blotting for S-glutathionylation

Western blotting under non-reducing conditions was performed using groups of 5 EDL fibre segments rather than single segments (see Methods), owing to the low sensitivity of anti-GSH antibody used to detect S-glutathionylation. An anti-GSH signal for a protein running at  $\sim 23$  kDa was observed for all fibre samples subjected to the standard DTDP-GSH treatment, with little matching signal apparent in untreated (control) fibres or in fibres treated with DTT (e.g. Fig 4A & B). Reprobing the membranes with anti-TnI showed the 23 kDa band corresponded with the position of the fast isoform of TnI ( $\text{TnI}_f$ ) in every case. The density of the anti-GSH signal for  $\text{TnI}_f$  (normalized to the corresponding  $\text{TnI}_f$  density) was  $\sim 4$  times greater in the DTDP-GSH treated fibres relative to the control fibres (Fig. 4C) fibres; these data were obtained from a total of 11 independent gels and DTDP-GSH treated samples, comparing matching control samples collected in the same experiments in 10 of these cases. The anti-GSH/ $\text{TnI}_f$  ratios for other treatment samples run on a subset of the same gels, expressed relative to DTDP-GSH case (defined as '1'), were  $0.38 \pm 0.26$  (s.d.,  $n=2$ ) for samples treated with just DTT, and  $0.53 \pm 0.07$  ( $n=4$ ) for fibres treated with DTDP-GSH and then DTT, and  $0.31 \pm 0.18$  ( $n=3$ ) for fibres pretreated with NEM and DTT before DTDP-GSH. The latter two cases were significantly lower than for the DTDP-GSH treatment alone (Fig. 4C), consistent with DTT reversing the effect of DTDP-GSH treatment and with NEM blocking its action. Treatment of fibres with GSSG-pH 8.5 also increased the anti-GSH signal relative to control. In the two cases examined, the anti-GSH/ $\text{TnI}_f$  ratio expressed relative to the DTDP-GSH case run on the same gel was  $0.3 \pm 0.1$  for the control treatment and  $2.0 \pm 0.2$  for the GSSG-pH 8.5 treatment.

Most membranes were also reprobbed for actin, and it was found that the TnI<sub>f</sub> signal expressed relative to either the actin signal or the MHC signal (see Methods) was not significantly altered by any of the treatments. The anti-GSH blots invariably also showed strong signals corresponding to actin (e.g. Fig. 4A), but these signals were not noticeably different between the control and treatment cases. The only other band where the anti-GSH signal was often evidently more intense for the DTDP-GSH treated samples relative to control was seen at ~100 kDa and possibly corresponded to the SR Ca-ATPase, which is known to undergo S-glutathionylation (Viner *et al.*, 1999).

Additional experiments were also performed that avoided any possible problems arising with reprobbed of membranes (e.g. probing first with anti-GSH and then with anti-TnI, as in Figure 4). In these experiments, treated EDL fibre samples were divided in three equal parts, which were run on three separate gels, with one each being first probed for either anti-GSH, anti-TnI or anti-TnC. These experiments (not shown) gave results indistinguishable from those obtained by the reprobbed procedure, demonstrating that the anti-TnI<sub>f</sub> signal was accurately assessed by the reprobbed method and further verifying that the DTDP-GSH treatment increases the anti-GSH/TnI<sub>f</sub> ratio. Importantly too, neither type of blotting procedure (e.g. Fig 4 A & B) found any apparent anti-GSH signal corresponding to either TnC<sub>f</sub> or myosin light chain 2 (MLC), which both run at ~18 kDa. (Note that fast TnT isoforms were not examined as they do not contain cysteine residues and hence cannot undergo S-glutathionylation). The experiments also found no evidence of any appreciable cross-linking of TnI<sub>f</sub> and TnC<sub>f</sub> either to each other or to actin, as only single bands were found when probing for TnI or TnC (at ~23 and 18 kDa, respectively). In summary, both western blotting methods indicated that the DTDP-GSH and GSSG-pH 8.5 treatments cause S-glutathionylation of TnI<sub>f</sub>.

### **Biotin labeling of reactive cysteine residues**

The reactivity of the free cysteine groups present in an EDL muscle fibre in relaxing conditions was examined using a reactive thiopyridine reagent (Biotin-HPDP) with a biotin tag that can be detected with high sensitivity. As seen in Figure 5, a one minute exposure to the reagent produced appreciable biotin labeling of TnI<sub>f</sub> as well as labeling of actin and a protein running at ~105 kDa (possibly the SR Ca-ATPase). Longer exposure to the reagent gave stronger labeling of these proteins as well as of MHC and some other proteins. Taking into account the relative amounts of each protein present in a muscle fibre, the comparatively strong labeling to TnI<sub>f</sub> seen with the 1 min exposure to Biotin-HPDP confirms that one or more cysteine residues present on TnI<sub>f</sub> in situ on the thin filament in relaxing conditions is exposed and relatively reactive to thiopyridine reagents. Furthermore, the absence of any biotin labeling at ~18 kDa even with the more prolonged reagent exposure indicates that the cysteine residues present in TnC<sub>f</sub> and MLC2 are inaccessible or comparatively unreactive in a fibre in relaxing conditions.

### **S-glutathionylation of TnI<sub>f</sub> by GSNO**

GSNO can potentially cause either S-glutathionylation or S-nitrosylation of a given cysteine residue. We have previously reported (Dutka *et al.*, 2011b) that when GSNO (2 mM) is applied to a skinned EDL fibre immediately after being prepared in solution (termed GSNO<sub>imm</sub>) it causes a large increase in Ca<sup>2+</sup>-sensitivity (~+0.14 pCa units), seemingly by the same mechanism as occurs with DTDP-GSH treatment. In contrast, if GSNO is applied to the fibre ~10 min after preparation (termed GSNO<sub>del</sub>) it instead causes a decrease in Ca<sup>2+</sup> sensitivity (~ -0.06 pCa units), seemingly in a manner similar to the S-nitrosylating agent, SNAP. As seen in Figure 6, western blotting showed that GSNO<sub>imm</sub> caused S-glutathionylation of TnI<sub>f</sub>, whereas GSNO<sub>del</sub> treatment did not. Very similar results were found in all three independent examinations conducted.

### **Troponin exchange experiments**

To further examine whether S-glutathionylation of TnI<sub>f</sub> is mechanism underlying the increase in Ca<sup>2+</sup>-sensitivity, we tested whether substituting fast troponin isoforms into slow-twitch (type I) muscle fibres made them responsive to the DTDP-GSH treatment. The procedures attempted on rat skinned fibres were a) to extract just the endogenous TnC and then to add exogenous fast TnC from either porcine or rabbit muscle, b) to extract both TnC and TnI and then to add the exogenous fast isoforms, or c) to substitute the endogenous TnC and TnI (and probably TnT) without first going through a specific extraction step (see Methods).

*TnC extraction and re-addition.* TnC extraction was achieved by exposing a skinned fibre for 30 min to a rigor solution of normal ionic strength with TFP and no Ca<sup>2+</sup> or Mg<sup>2+</sup>. This resulted in complete loss of Ca<sup>2+</sup>-activated force in the 8 EDL type II fibres and its reduction to between 1 - 4% of the original level in the 4 soleus type I fibres examined, with no change in baseline Ca<sup>2+</sup>-independent force. Western blotting in subset of these and other fibres confirmed extraction of 80 to 100% of the TnC, with relatively little if any change in TnI (not shown). After the fibres had been bathed for 15 min in relaxing solution containing exogenous fast troponins (i.e TnC, TnI and TnT) from either rabbit or porcine muscle, maximum force recovered to 56 ± 3% and 39 ± 1% of its original value in type II (n=6) and type I (n=4) fibres respectively. Almost exactly the same level of recovery was seen in two type II fibres when adding TnC<sub>f</sub> alone, indicating that the reduction in maximum force had been primarily due to the loss of TnC. Following the extraction and replacement of TnC, the Ca<sup>2+</sup>-sensitivity of the fibres was substantially altered: pCa<sub>50</sub> decreased by ~0.21 to 0.26 pCa units in the type II fibres and by ~0.35 to 0.44 pCa units in the type I fibres and *h* decreased ~50% in all cases, with no apparent difference between the porcine and rabbit TnC<sub>f</sub>. The pCa<sub>50</sub> is normally ~0.16 pCa units higher in rat type I fibres compared to type II fibres (Trinh & Lamb, 2006), but after substituting TnC<sub>f</sub> into both fibres types this difference disappeared.

Following the TnC<sub>f</sub> substitution, DTDP-GSH treatment increased pCa<sub>50</sub> by  $+0.187 \pm 0.026$  pCa units in the 4 type II fibres examined, demonstrating that its normal action still occurred. In contrast, in two other type II fibres where the endogenous TnC was extracted and the fibre exposed to 25  $\mu$ M NEM for 2 min before washing and adding exogenous TnC<sub>f</sub>, subsequent DTDP-GSH treatment caused only ~5% of its usual increase in Ca<sup>2+</sup>-sensitivity shift ( $+0.011 \pm 0.002$  SD), indicating that type II fibres were affected by NEM treatment even when TnC<sub>f</sub> was largely absent. In the type I fibres following TnC<sub>f</sub> substitution, DTDP-GSH treatment still failed to cause any significant change in pCa<sub>50</sub> ( $+0.021 \pm 0.007$ , n=4, P>0.05); western blotting of one of these type I fibres verified that most (though not all) of the slow TnC had indeed been replaced with the fast isoform.

*TnI and TnC extraction and re-addition.* Combined extraction of both TnC and TnI (and possibly some TnT) was achieved by bathing a fibre in relaxing solution with 10 mM vanadate for 10 min (Kogler *et al.*, 1998). No force was produced in the presence of the vanadate, but upon washout with relaxing solution force developed over several minutes in a Ca<sup>2+</sup>-independent manner, reaching a peak of  $31 \pm 3\%$  of the original Ca<sup>2+</sup>-activated maximum force in type II fibres (n=12) and  $62 \pm 6\%$  in type I fibres (n=7). This Ca<sup>2+</sup>-independent force was suggestive of at least partial TnI extraction, and western blotting of some fibres confirmed such partial extraction (not shown). Subsequent exposure of the fibre either to a mixture of TnC, TnI and TnT (fast isoforms), or to just TnI and TnT, resulted in the Ca<sup>2+</sup>-independent force declining to zero in the type II fibres and to ~2 to 6% of the original maximum Ca<sup>2+</sup>-activated force in the type I fibres. Ca<sup>2+</sup>-activated force only recovered if TnC was added with the TnI and TnT or alternatively added subsequently. Maximum force recovered to ~55 to 60% of the original level in the 3 type II fibres examined, but to only  $17 \pm 3\%$  in the type I fibres (n=7), which along with the partial nature of the troponin exchange, precluded meaningful examination of the effects of DTDP-GSH treatment in such fibres.

*Troponin substitution without prior extraction.* As extraction and subsequent replacement of the troponins appeared to lead to poor recovery of maximum force, troponin exchange was instead performed by exposing the skinned fibres to the exogenous troponins in the presence of a low ionic strength solution with zero  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (see Methods); such low ionic strength likely aids unbinding of TnT, allowing it to also be substituted, and possibly even allowing exchange of the entire endogenous slow troponin trimer complex with the exogenous fast troponin complex. Following 60 min of such exposure, maximum  $\text{Ca}^{2+}$ -activated force back in standard conditions was still 92 to 96% of the original maximum in the 5 type I rat fibres examined (3 with porcine and 2 with rabbit troponin exposure).

However, in contrast to what occurred when exchanging just  $\text{TnC}_s$  with  $\text{TnC}_f$  (where  $\text{pCa}_{50}$  was decreased after extraction and addition using *either* rabbit or porcine  $\text{TnC}_f$  (see above)), exchanging the TnI/TnT by either the vanadate treatment-replacement protocol or the low ionic strength exchange, resulted in a *decrease* in  $\text{Ca}^{2+}$ -sensitivity when adding rabbit troponins ( $\text{pCa}_{50} -0.12 \pm 0.03$  in 5 type I fibres and  $-0.20 \pm 0.02$  in 11 type II fibres) but a large *increase* when adding porcine troponins ( $\text{pCa}_{50} +0.47 \pm 0.03$  in 5 type I fibres and  $+0.17 \pm 0.04$  (s.d.) in 2 type II fibres). Such data are consistent with the fact that  $\text{TnC}_f$  is virtually identical in rat, rabbit and pig, whereas the TnI<sub>f</sub> isoforms present differ slightly between the three species, as well as the fact that at least three distinct variants of fast TnT are found in type II fibres even in a single species (Brotto *et al.*, 2006).

Most importantly, following substitution of TnI<sub>f</sub> into soleus type I fibres, DTDP-GSH treatment resulted in an increase in  $\text{Ca}^{2+}$ -sensitivity, as seen in Figure 7A & B. In this experiment the force- $\text{pCa}$  characteristics were examined after applying the DTDP and then again after applying GSH, in order to remove the confounding effects of the DTDP treatment itself and directly determine whether the GSH exposure caused a reversible increase in sensitivity. In the 3 type I fibres

examined following porcine TnI<sub>f</sub> substitution, GSH treatment caused a substantial increase in Ca<sup>2+</sup>-sensitivity (~ +0.126 pCa units), which was fully reversed by DTT (Table 2), whereas normal type I fibres show no significant effect to such treatment (results here and also Lamb & Posterino (2003) – see Table 2), with one of these control cases being one of the three fibres subsequently shown to undergo a large increase in Ca<sup>2+</sup>-sensitivity after troponin exchange. Western blotting of the type I fibre shown in Figure 7A indicated that ~35% of TnI<sub>s</sub> originally present in the fibre had indeed been substituted with TnI<sub>f</sub> (lane 2 in Figure 7C). This estimate of percentage exchange was based on the evident reduction in the intensity of the TnI<sub>s</sub> band in lane 2 relative to the bracketing untreated fibres run on either side (lanes 1 & 3) (taking into account the sample mass in each lane indicated by the MHC bands shown at the top), together with the fact that non-linearity in the signal detection of the bands evidently resulted in disproportionately weak signals for smaller TnI amounts (which also likely precluded detection of any substituted TnI<sub>f</sub> in the relatively small sample in lane 4) (see also (Murphy *et al.*, 2009)). The reversible increase in Ca<sup>2+</sup>-sensitivity with DTDP-GSH treatment also appeared to occur in the two type I fibres examined following exchange with rabbit troponin (Table 2), albeit to a smaller extent than with the porcine troponin exchange.

### **Effects of DTDP-GSH treatment in human muscle fibres**

The effect of DTDP-GSH treatment was also examined in human *vastus lateralis* muscle fibres. Each fibre was classified as either type I or II both by its response to Sr<sup>2+</sup> (see Methods) and by subsequent western blotting for MHC, TnC and TnI (e.g. Fig 8B), the results of which were in complete accord for each of the 5 fibres examined. **All three of the human type II fibres examined (obtained from three different subjects) showed a large increase in Ca<sup>2+</sup>-sensitivity with DTDP-GSH treatment (~ +0.171 pCa units, row 8 in Table 1), which was fully reversed by DTT (e.g. Fig 8A).** In contrast, none of the 3 type I human fibres examined showed any increase in Ca<sup>2+</sup>-sensitivity to DTDP-GSH, instead displaying a decrease in every case (mean change in pCa<sub>50</sub> of -0.111 ± 0.006 pCa units,) with subsequent DTT treatment increasing Ca<sup>2+</sup>-sensitivity (+0.053 ±



0.014 pCa units); this effect of the DTDP-GSH treatment was largely or entirely attributable to the effects of the DTDP treatment alone, as seen in Figure 8C. The 5 min DTDP treatment appeared to have greater effects in human fibres than it did in rat fibres, reversibly decreasing maximum force by 5 to 15%, which was similar to that seen in rat fibres with more prolonged treatments or higher [DTDP] (see (Lamb & Posterino, 2003)). In summary, the results with human muscle closely resemble those in rat, with only the type II fibres showing increased  $\text{Ca}^{2+}$ -sensitivity to the DTDP-GSH treatment.

TnI<sub>f</sub> in human muscle is highly homologous to that in other mammals, and as seen in Figure 9A it also undergoes S-glutathionylation upon DTDP-GSH treatment. The slow isoform of TnI, TnI<sub>s</sub>, is also S-glutathionylated by the DTDP-GSH treatment in both human muscle (Fig 9A, see presence of two bands with anti-GSH) and rat soleus muscle (not shown). Figure 9A also illustrates two other important points. Firstly, the TnI<sub>f</sub> in rested human muscle shows some base level of S-glutathionylation (see lane 3). Secondly, S-glutathionylation of TnI<sub>f</sub> causes the TnI<sub>f</sub> to run slightly slower (i.e. at slightly higher apparent molecular mass) in both rat (lane 2 versus 1) and human muscle (lane 4 versus 3). Close inspection of all gels where TnI<sub>f</sub> underwent S-glutathionylation consistently showed this small effect (e.g. Figs 4, 5, 6 & 10), though it was most apparent when precisely matched samples were compared as in Figure 9A. One result of this is that when only a portion of the total TnI<sub>f</sub> in a sample is S-glutathionylated, the latter forms the trailing (or upper) part of the TnI<sub>f</sub> band seen with anti-TnI (e.g. lower panel in Fig. 9A), and because only it is seen with anti-GSH (middle panel in Fig 9A) the anti-GSH bands for TnI<sub>f</sub> and TnI<sub>s</sub> appear closer together than do the anti-TnI bands for the two proteins. (Note that this effect is in part due to the fact that TnI<sub>s</sub> evidently does not run any slower when glutathionylated). Importantly, the fact that the TnI<sub>f</sub> band can be seen to run slower following the DTDP-GSH treatment is further strong evidence that the TnI<sub>f</sub> is indeed affected by the treatment and is correctly identified as being the S-glutathionylated protein apparent on the anti-GSH blots.

### **Effect of DTDP-GSH treatment in toad and chicken type II fibres**

TnI<sub>f</sub> in most if not all mammalian species has three cysteine residues, Cys48, Cys64 and Cys133, and in the troponin complex only Cys133, which is highly reactive, is accessible to applied reactants (Chong & Hodges, 1982), as illustrated in Figure 9B. Therefore it can be presumed that the S-glutathionylation of TnI<sub>f</sub> occurs at Cys-133. Consistent with this, mammalian TnI<sub>s</sub> lacks an equivalent of Cys133; it has a Cys65 residue homologous to Cys64 in TnI<sub>f</sub>, as well as two other cysteine residues (Cys38 and Cys85) that are quite distinct from those in TnI<sub>f</sub>. The reactivity and accessibility of the cysteine residues in TnI<sub>s</sub> have not been described, but the results here indicate that at least one of the residues, possibly Cys38 or Cys85, is accessible and becomes S-glutathionylated with DTDP-GSH treatment.

TnI<sub>f</sub> in chicken and toad muscle are highly homologous to mammalian TnI<sub>f</sub> except that they both lack the equivalent of Cys133 (Wilkinson & Grand, 1978). Type II fibres were obtained from iliofibularis muscle of the toad and the pectoralis major muscle of the chicken and all showed the expected low sensitivity of Sr<sup>2+</sup> (O'Connell *et al.*, 2006) and fast TnC and TnI bands on western blotting (not shown). Importantly, DTDP-GSH treatment caused no significant change in Ca<sup>2+</sup>-sensitivity in either the chicken or toad type II fibres (rows 9 and 10 in Table 1), consistent with the increased Ca<sup>2+</sup>-sensitivity occurring in mammalian muscle being due to S-glutathionylation of Cys133. (In the chicken and toad fibre experiments listed in Table 1, the DTDP exposure was reduced from 5 min to 1 min, because the longer exposure to DTDP itself resulted in a moderate reduction in maximum force and Ca<sup>2+</sup>-sensitivity particularly in the chicken fibres; importantly, GSH exposure following the longer DTDP treatment still did not cause any increase in Ca<sup>2+</sup>-sensitivity in any of the fibres examined).

Furthermore, DTDP-GSH treatment did not cause any S-glutathionylation of TnI<sub>f</sub> in toad type II fibres, as seen in Figure 10. Note that the anti-GSH band detected in the toad fibres in a similar

position to TnI<sub>f</sub> in the rat fibres is an unknown protein and does not correspond to the TnI<sub>f</sub> in the toad fibres, which was shown on the TnI reprobe to run somewhat lower. The full anti-GSH blot presented in Figure 10 shows that there are a number of S-glutathionylated proteins present in the toad fibres that are not present in the rat fibres. Importantly, it also shows that the TnI<sub>f</sub> was the only protein present in these rat type II fibres with a noticeable change in S-glutathionylation upon DTDP-GSH treatment.

### **S-glutathionylation of TnI<sub>f</sub> in exercising humans**

Finally, we examined whether the level of S-glutathionylation of TnI<sub>f</sub> in human muscle was affected by exercise (see Methods). We found that there was a ~4 fold increase in the level of S-glutathionylation of TnI<sub>f</sub> in *vastus lateralis* muscle of 5 human subjects following 40 min of cycling exercise at ~60%  $V_{O2,peak}$  (Fig 11).

## Discussion

This study provides compelling evidence that TnI<sub>f</sub> in mammalian fast-twitch (type II) fibres can be readily S-glutathionylated and that this results in a relatively large increase in Ca<sup>2+</sup>-sensitivity of the contractile apparatus with no detectable change in maximum force. The maximal effect was a ~ +0.24 pCa unit increase in pCa<sub>50</sub> (Table 1), which corresponds to a 1.7 fold decrease in [Ca<sup>2+</sup>] needed for half-maximal force. We previously showed that treating rat type II fibres with DTDP and then briefly (1 to 2 min) with GSH produces this increase in Ca<sup>2+</sup>-sensitivity, and that this could be reversed by DTT or much longer (>20 min) exposure to GSH, effects, strongly suggesting that the key process involved S-glutathionylation of some unidentified contractile apparatus protein (Lamb & Posterino, 2003). It was found here that a 10 min exposure to oxidized glutathione (GSSG) had the same action, and produced approximately the same maximal effect if applied at pH 8.5 (Fig.s 1 & 2, Table 1), the latter being used as a means of increasing the reactivity of the cysteine residues (Klatt & Lamas, 2000; Dalle-Donne *et al.*, 2007). Western blotting showed that both the DTDP-GSH and GSSG-pH 8.5 treatments caused S-glutathionylation of TnI<sub>f</sub> in every instance examined (e.g. Fig.s 4, 9 & 10), without having any apparent effect on the other possible target proteins, in particular TnC<sub>f</sub> or myosin light chain 2. The only other protein seen in many instances to display increased S-glutathionylation with the DTDP-GSH treatment was a protein of ~100 kDa, which was likely to be the SR Ca<sup>2+</sup> pump (Viner *et al.*, 1999). Effects on the Ca<sup>2+</sup> pump could not account for the increased sensitivity of the contractile apparatus, particularly given that the increased Ca<sup>2+</sup>-sensitivity still occurred even after removal of all membranes with the detergent Triton-X100. Western blotting further demonstrated that there was no appreciable level of cross-linking of TnI<sub>f</sub> with TnC<sub>f</sub> or other proteins upon DTDP-GSH treatment. Treatment with GSNO can cause either increased or decreased Ca<sup>2+</sup>-sensitivity depending on how it is applied (Dutka *et al.*, 2011b), and it was found here that the increase in Ca<sup>2+</sup>-sensitivity occurred only when TnI<sub>f</sub> was S-glutathionylated (Fig. 6). Finally, DTT treatment reversed both the increase in Ca<sup>2+</sup>-sensitivity

and the S-glutathionylation of TnI<sub>f</sub>, and NEM pre-treatment blocked both effects (Figs 3 & 4 & Table 1).

Type I fibres lack TnI<sub>f</sub>, instead having the slow isoform TnI<sub>s</sub>, and these fibres show no increase in Ca<sup>2+</sup>-sensitivity to either DTDP-GSH treatment or GSSG-pH 8.5 treatment (Table 1). Crucially, after TnI<sub>s</sub> was partially substituted with TnI<sub>f</sub>, the type I fibres did show increased Ca<sup>2+</sup>-sensitivity upon DTDP-GSH treatment (Fig. 7 & Table 2), strongly implicating TnI<sub>f</sub> as the crucial mediator of the effect. The effects could not have been mediated by TnT<sub>f</sub>, because there are no cysteine residues in any of the three mammalian variants of TnT<sub>f</sub> and hence they cannot undergo S-glutathionylation. It was also apparent that the effects were not mediated by TnC<sub>f</sub>, because i) as mentioned above, TnC<sub>f</sub> did not become S-glutathionylated with DTDP-GSH treatment, ii) the Ca<sup>2+</sup>-sensitivity increase with DTDP-GSH treatment takes place even when a fibre is maximally activated by Ca<sup>2+</sup>, which is known to make the one cysteine residue on TnC<sub>f</sub> (Cys98) inaccessible (Park *et al.*, 1994), iii) specific extraction of TnC<sub>s</sub> and replacement with TnC<sub>f</sub> did not cause type I fibres to display increased Ca<sup>2+</sup>-sensitivity with DTDP-GSH, and iv) type II fibres treated with NEM whilst largely devoid of TnC<sub>f</sub> showed virtually no Ca<sup>2+</sup>-sensitivity increase to DTDP-GSH following re-insertion of TnC<sub>f</sub>. Furthermore, the biotin-HPDP labeling experiments (e.g. Fig. 5) indicated that the cysteine on TnC<sub>f</sub> was inaccessible or poorly reactive to thiopyridine reagents.

### **Cysteine target on TnI<sub>f</sub>**

Mammalian TnI<sub>f</sub> has three cysteine residues (see Fig. 9B), but when TnI<sub>f</sub> is in the troponin complex with TnC and TnT only Cys133 is accessible and reactive, and this is the case in both the presence and absence of Ca<sup>2+</sup> (Chong & Hodges, 1982). Thus, the S-glutathionylation of TnI<sub>f</sub> observed here is only attributable to an action on Cys133. Furthermore, the finding here that the increase in Ca<sup>2+</sup>-sensitivity with DTDP-GSH treatment was the same in both resting and activated fibres, is fully consistent with the known accessibility of Cys133. The fact that the sensitivity shift to DTDP-GSH

treatment remained the same after replacement of TnI<sub>f</sub> in rat type II fibres with either porcine or rabbit TnI<sub>f</sub> is also consistent with Cys133 being the key target, as it is present in all these variants of TnI<sub>f</sub>. This cysteine residue is likewise present in the TnI<sub>f</sub> of human type II fibres, which also show the Ca<sup>2+</sup>-sensitivity increase to DTDP-GSH (Fig. 8A). Type II fibres from toad and chicken, on the other hand, have fast isoforms of TnI and TnC that are highly homologous to mammalian TnI<sub>f</sub> and TnC<sub>f</sub>, having matching cysteine residues to Cys98 in TnC<sub>f</sub>, and to Cys 48 and Cys64 in TnI<sub>f</sub>, but they lack the equivalent of Cys133, having an asparagine residue in its place (Wilkinson & Grand, 1978). Significantly, neither toad nor chicken type II fibres showed any change in Ca<sup>2+</sup>-sensitivity with DTDP-GSH treatment (Table 1), and western blotting of TnI<sub>f</sub> in toad fibres showed no detectable S-glutathionylation, either with or without DTDP-GSH treatment (Fig. 10). These findings together strongly implicate S-glutathionylation of Cys133 on TnI<sub>f</sub> as being the process responsible for the increased Ca<sup>2+</sup>-sensitivity occurring with DTDP-GSH treatment.

Cys133 on TnI<sub>f</sub> is located in a highly flexible and mobile domain of the protein which can oscillate back and forth between actin and TnC (Aihara *et al.*, 2006; Aihara *et al.*, 2010). The segment on TnI<sub>f</sub> adjacent to Cys133 binds to the hydrophobic pocket in the N-lobe of TnC in the Ca<sup>2+</sup> bound state, and swings back to be frequently near actin in the absence of Ca<sup>2+</sup>. With S-glutathionylation, the glutamate residue of glutathione adds a negative charge at the cysteine residue, and this together with the accompanying steric effects is thought to exert an action similar to or even greater than occurs with protein phosphorylation (Klatt & Lamas, 2000; Dalle-Donne *et al.*, 2007). It seems that the net effect of this is to bias the movements of the flexible Cys133 region of TnI<sub>f</sub> more towards the TnC bound state, so that the interaction between TnI and TnC that controls contractile activation occurs at lower cytoplasmic [Ca<sup>2+</sup>].

Interestingly, although TnI<sub>s</sub> does not have a cysteine residue matching Cys133 in TnI<sub>f</sub>, it does have a serine residue in the corresponding position (Ser134), raising the possibility that phosphorylation

of TnI<sub>s</sub> at Ser134 may play a similar role in modifying the Ca<sup>2+</sup>-sensitivity of contraction in type I fibres. Furthermore, cardiac TnI (TnI<sub>c</sub>) also has a serine residue in the homologous location (Ser166 in rat and Ser165 in human), adjacent to region binding in the hydrophobic pocket of cardiac TnC<sub>c</sub>, and phosphorylation of this residue on TnI<sub>c</sub> impedes binding of TnI<sub>c</sub> to TnC<sub>c</sub> (Ward *et al.*, 2001). However, such phosphorylation does not occur when TnI<sub>c</sub> is in the normal troponin complex (Ward *et al.*, 2001). In view of the major effects on Ca<sup>2+</sup>-sensitivity found here with S-glutathionylation of Cys133 in TnI<sub>f</sub>, the intriguing structural parallels with TnI<sub>s</sub> and TnI<sub>c</sub> suggest that a detailed investigation of the occurrence and possible functional changes occurring with phosphorylation at these sites may be warranted.

### **Functional relevance of S-glutathionylation of TnI<sub>f</sub>**

S-glutathionylation of TnI<sub>f</sub> was produced in the present study primarily by unphysiological means, in particular by applying DTDP-GSH or a very high level of GSSG that would never be reached in a muscle cell. However, the same basic process could be expected to quite readily occur in muscle fibres. Cys133 of TnI<sub>f</sub> is evidently a highly reactive and accessible cysteine residue (results here and (Chong & Hodges, 1982; Tao *et al.*, 1990; Park *et al.*, 1994)) and hence could be expected to readily react with many of the variety of reactive oxygen and nitrogen species, and other related reactive species, generated during muscle activity (Reid *et al.*, 1992; Allen *et al.*, 2008; Powers & Jackson, 2008; Lamb & Westerblad, 2011), leading by various means to its S-glutathionylation in the presence of cytoplasmic GSH (see Introduction). The rate at which the cytoplasmic GSH subsequently reduces the glutathionylated cysteine residue on TnI<sub>f</sub> back to a free sulphhydryl was found previously to be relatively slow, with only ~50% reversal occurring in 20 min in the presence of 5 mM GSH (Lamb & Posterino, 2003). This would help favour accumulation of TnI<sub>f</sub> in the S-glutathionylated state. However, reversal may occur faster in-vivo at higher temperature and with the presence of glutaredoxins and thioredoxins (Dalle-Donne *et al.*, 2007).

Most importantly, the present study found that moderate intensity cycling exercise for 40 min led to a ~4-fold increase in S-glutathionylation of TnI<sub>f</sub> in the *vastus lateralis* muscle of human subjects (Fig. 11). Given that S-glutathionylation of TnI<sub>f</sub> of human type II fibres was found, like in rat type II fibres, to result in a large increase in Ca<sup>2+</sup>-sensitivity (Figs 8 & 9), it seems reasonable to conclude that S-glutathionylation of TnI<sub>f</sub> is likely to be a significant factor influencing muscle performance in exercising humans. Increasing the Ca<sup>2+</sup>-sensitivity of the contractile apparatus in this way could be of great benefit in countering factors occurring with normal exercise which decrease contractile sensitivity, such as the build-up of inorganic phosphate which is thought to be a major factor in causing muscle fatigue (Allen *et al.*, 2008).

S-glutathionylation of TnI<sub>f</sub> not only increases contractile Ca<sup>2+</sup>-sensitivity considerably but also increases the peak twitch force and rate of force development to action potential stimulation (see Fig 4 in (Dutka *et al.*, 2011b)). Thus, the overall effects of S-glutathionylation of TnI<sub>f</sub> are highly comparable to those occurring with phosphorylation of myosin light chain 2 (Szczesna *et al.*, 2002; Stull *et al.*, 2011), with both causing similar size increases in Ca<sup>2+</sup>-sensitivity and similar twitch force potentiation in fast-twitch muscle fibres, though by distinctly different mechanisms. It is interesting that the potentiating effects of myosin light chain phosphorylation are seen within a matter of seconds from onset of intense muscle stimulation (Stull *et al.*, 2011) and that the extent of MLC2 phosphorylation is greatly reduced after more prolonged fatiguing stimulation even though twitch potentiation still persists (Tubman *et al.*, 1996). The latter finding, together with that fact that fatiguing stimulation causes twitch potentiation even in myosin light chain kinase knockout mice has led to the suggestion that other mechanisms can also seemingly cause such post-activation potentiation when muscle has undergone a period of fatiguing stimulation (Tubman *et al.*, 1996; Stull *et al.*, 2011). Clearly, the findings of this study suggest that S-glutathionylation of TnI<sub>f</sub> would fulfill such a role, especially given that its effects would be expected to increase with the duration and extent of muscle activity owing to the likely increased total generation of reactive oxygen and



related species. However, if the activity continued for too long it is possible that other deleterious effects of the oxidants could antagonize and counter the potentiating effects of the S-glutathionylation of TnI<sub>f</sub> (Andrade *et al.*, 1998; Lamb & Westerblad, 2011).

### *Concluding remarks*

This study has shown that S-glutathionylation of TnI<sub>f</sub> readily occurs in fast-twitch skeletal muscle fibres, including in humans during prolonged strenuous cycling exercise. Such S-glutathionylation greatly increases the rate of force development and the Ca<sup>2+</sup>-sensitivity of the contractile apparatus, and such effects could be expected to substantially influence muscle performance, in particular helping counter the effects of other factors that decrease contractile sensitivity or sarcoplasmic reticulum Ca<sup>2+</sup> release that if unchecked potentially lead to reduced force responses and ‘muscle fatigue’. Additionally, the findings implicate Cys133 on TnI<sub>f</sub> as the site of the S-glutathionylation, and this seemingly fits well with its location in the flexible region of TnI<sub>f</sub> adjacent to the region binding to TnC during Ca<sup>2+</sup> activation. The identification of this molecular process could well provide new avenues for understanding muscle dysfunction in particular situations and possibly also for the development of novel therapeutic strategies for countering such dysfunction.

**Author contributions**

JPM and RMM designed, carried out and analyzed the western blotting procedures, and prepared the related figures and methods. TLD helped conceive and carry out some of the physiological studies on single fibres. CL and MJK were responsible for subject care and obtaining the muscle biopsies for the human skinned fibre data. TLM and GKM were responsible for the human exercise experiments and related muscle biopsies. GDL conceived and designed the overall project and the specific physiological and biochemical experiments, analyzed the physiological data and drafted the manuscript. All authors have read the final version of the submitted manuscript. Biochemical and physiological measurements were performed at La Trobe University and the human exercise procedures and muscle biopsies performed at Victoria University and University of Melbourne.

**Acknowledgments**

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**Table 1. Changes in  $Ca^{2+}$  sensitivity with glutathionylation treatments in type II and type I fibres.**

Data are mean  $\pm$  s.e.m. (or s.d., where n=2) of the change in  $pCa_{50}$  caused by indicated treatment, and by subsequent DTT treatment in a subset of those fibres. Each treatment applied with skinned fibre in the reduced state, except for 'Post-NEM' cases, where the fibre was pre-treated with 25  $\mu$ M NEM for 2 min. Treatments (all applied at  $pCa$  9): 100  $\mu$ M DTDP for 5 min (or 1 min in chicken and toad) followed by 5 mM GSH for 2 min; or 10 mM GSSG at pH 7.1 for 10 min; or 10 mM GSSG at pH 8.5 for 10 min. DTT treatment: 10 mM for 10 min in all cases. Number of fibres shown in brackets. N.D., not determined. Fibres classified as type I or II by  $Sr^{2+}$  activation properties, and verified by western blotting. \* denotes significantly different from zero ( $P < 0.05$ ); samples with n=2 not statistically compared.

<u>Treatment</u>	<u><math>\Delta pCa_{50}</math> after treatment</u>	<u><math>\Delta pCa_{50}</math> after DTT</u>
	A	B
<u>Rat Type II</u>		
1) DTDP-GSH	+0.238 $\pm$ 0.005 (18) *	-0.240 $\pm$ 0.004 (11) *
2) DTDP-GSH (Post-NEM)	+0.030 $\pm$ 0.007 (8) *	-0.045 $\pm$ 0.004 (5) *
3) GSSG pH 7.1	+0.055 $\pm$ 0.010 (3) *	N.D.
4) GSSG pH 8.5	+0.191 $\pm$ 0.009 (7) *	-0.218 $\pm$ 0.003 (2)
5) GSSG pH 8.5 (Post-NEM)	-0.003 $\pm$ 0.004 (2)	N.D.
<u>Rat Type I</u>		
6) DTDP-GSH	-0.018 $\pm$ 0.008 (7)	-0.009 $\pm$ 0.003 (6) *
7) GSSG pH 8.5	-0.023 $\pm$ 0.010 (2)	-0.015 $\pm$ 0.001 (2)
<u>Human Type II</u>		
8) DTDP-GSH	+0.171 $\pm$ 0.009 (3) *	-0.196 $\pm$ 0.012 (3) *
<u>Toad Type II</u>		
9) DTDP-GSH	-0.010 $\pm$ 0.006 (5)	+0.006 $\pm$ 0.008 (4)
<u>Chicken Type II</u>		
10) DTDP-GSH	-0.051 $\pm$ 0.013 (3)	+0.001 $\pm$ 0.009 (2)

**Table 2. Effect of glutathionylation after substituting fast troponin isoforms into slow-twitch fibres.** Mean  $\pm$  s.e.m. (or s.d., where n=2) of change in pCa<sub>50</sub> occurring with the indicated treatment in slow-twitch (i.e. type I) fibres from rat soleus muscle. n is the number of fibres examined. Treatments applied sequentially in order left to right across table. Substituted fast troponins were from porcine or rabbit muscle as indicated. N.A., not applicable. 'L& P, 2003' denotes data from Lamb & Posterino (2003), carried out with same conditions as this study. \* significantly different from zero (P<0.05).

	<b>Treatment</b>			
	<b>Troponin Exch.</b>	<b>DTDP</b>	<b>GSH</b>	<b>DTT</b>
		<b>A<sub>1</sub></b>	<b>A<sub>2</sub></b>	<b>B</b>
<b>1) Control soleus fibres</b> (L & P, 2003) (n=5)	N.A.	-0.059 $\pm$ 0.0027	+0.001 $\pm$ 0.002	+0.023 $\pm$ 0.011
<b>2) Control soleus fibres</b> This study (n=2)	N.A.	-0.028 $\pm$ 0.001	+0.012 $\pm$ 0.004	-0.004 $\pm$ 0.017
<b>3) Soleus fibres with porcine Troponin</b> This study (n=3)	+0.455 $\pm$ 0.030 *	-0.094 $\pm$ 0.016 *	+0.126 $\pm$ 0.027 *	-0.134 $\pm$ 0.022 *
<b>4) Soleus fibres with rabbit Troponin</b> This study (n=2)	-0.047 $\pm$ 0.001	-0.006 $\pm$ 0.028	+0.031 $\pm$ 0.006	-0.069 $\pm$ 0.043

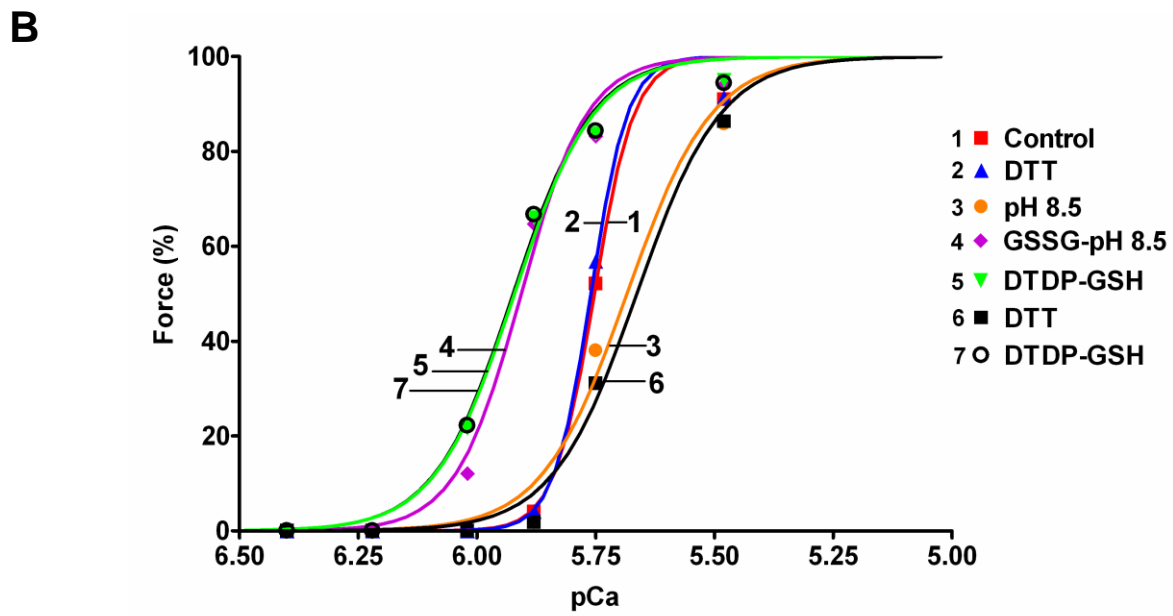
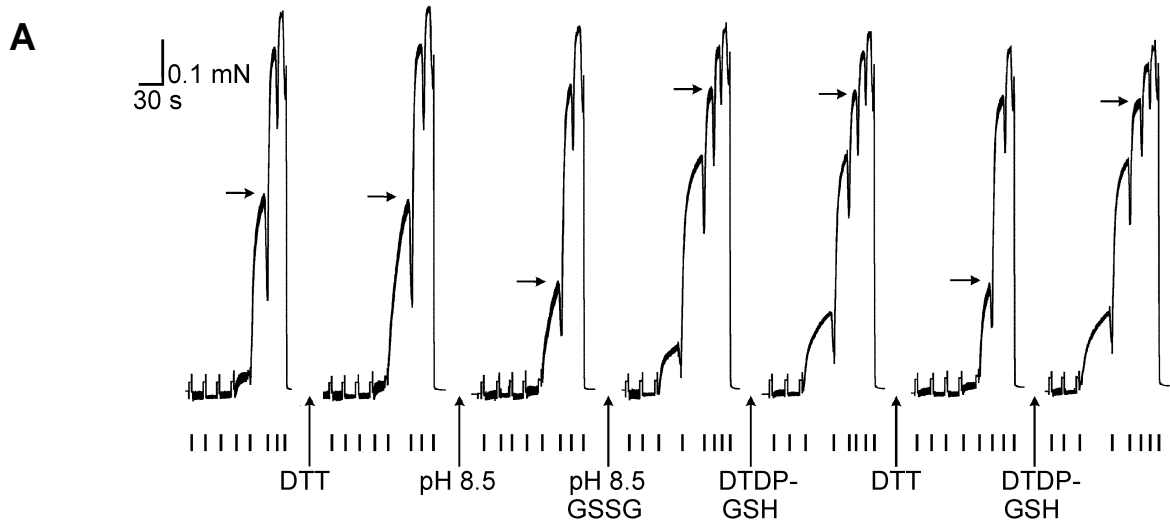
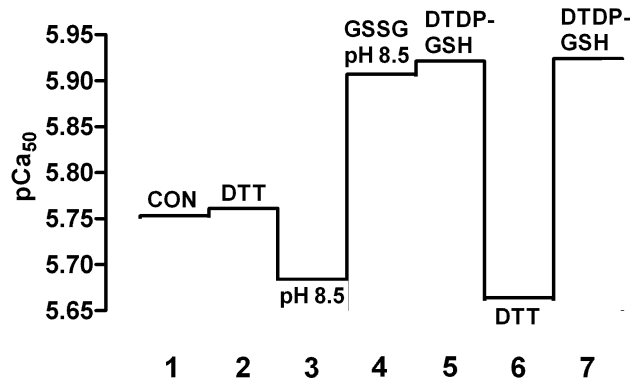


Figure 1. (see over)

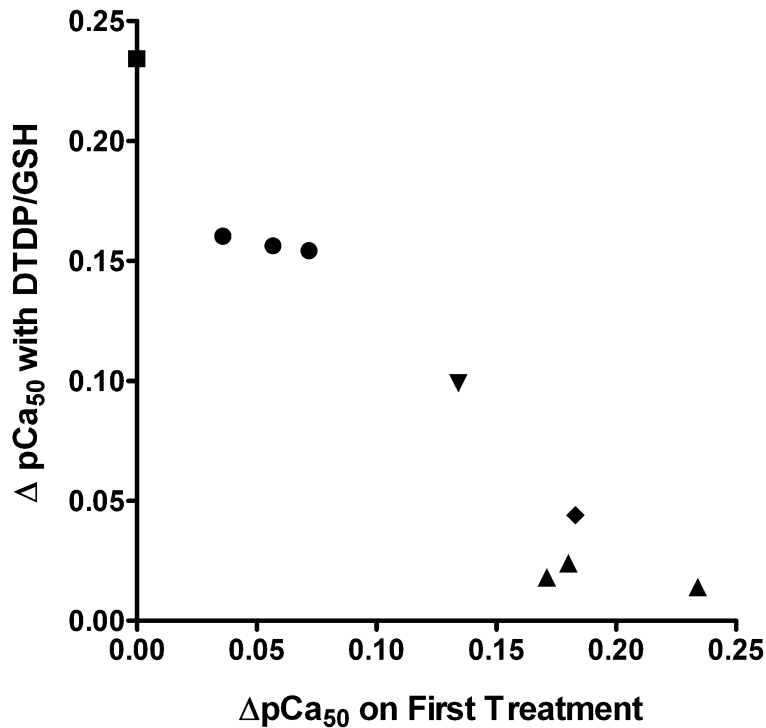
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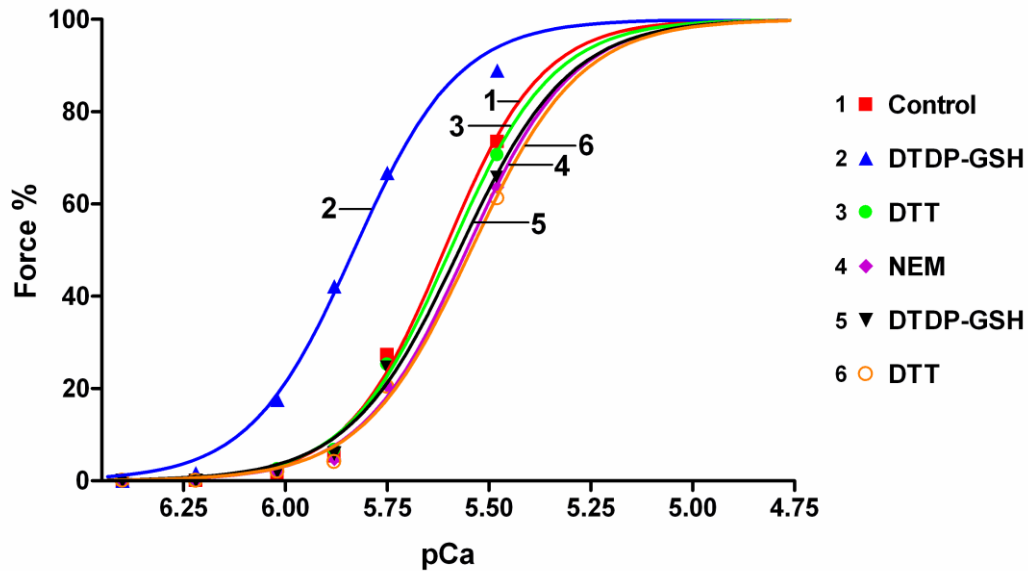
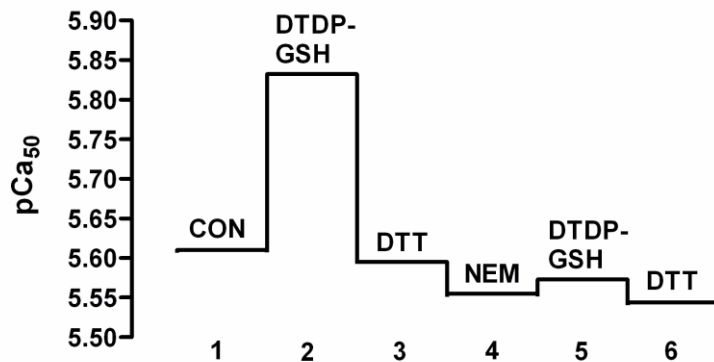
**Figure 1. Treatment with GSSG-pH 8.5 increases  $\text{Ca}^{2+}$  sensitivity of contractile apparatus.**

**A**, Isometric force production in a skinned EDL fibre to solutions with successively higher free  $[\text{Ca}^{2+}]$ , starting at  $\text{pCa} > 9$  (small ticks in order:  $\text{pCa}$  6.40, 6.22, 6.02, 5.88, 5.75, 5.48, 4.7,  $> 9$ ). Sequence repeated twice after each treatment, giving very similar results; only the second of each pair of force- $\text{pCa}$  staircases is shown for each treatment. Maximum  $\text{Ca}^{2+}$ -activated force reached at  $\text{pCa}$  4.7.

Horizontal arrows mark force level achieved at  $\text{pCa}$  5.75. Treatments (all applied at  $\text{pCa} > 9$ ): 10 mM DTT for 10 min; pH 8.5 for 10 min; 10 mM GSSG at pH 8.5 for 10 min; 100  $\mu\text{M}$  DTDP for 5 min followed by 5 mM GSH for 2 min (i.e. ‘standard DTDP-GSH treatment’). **B**, Hill fits to force- $\text{pCa}$  data following each indicated treatment; data from panel A, with force responses in each ‘staircase’ normalised to their own maximum. **C**,  $\text{pCa}_{50}$  values after indicated treatment for Hill fits in B.

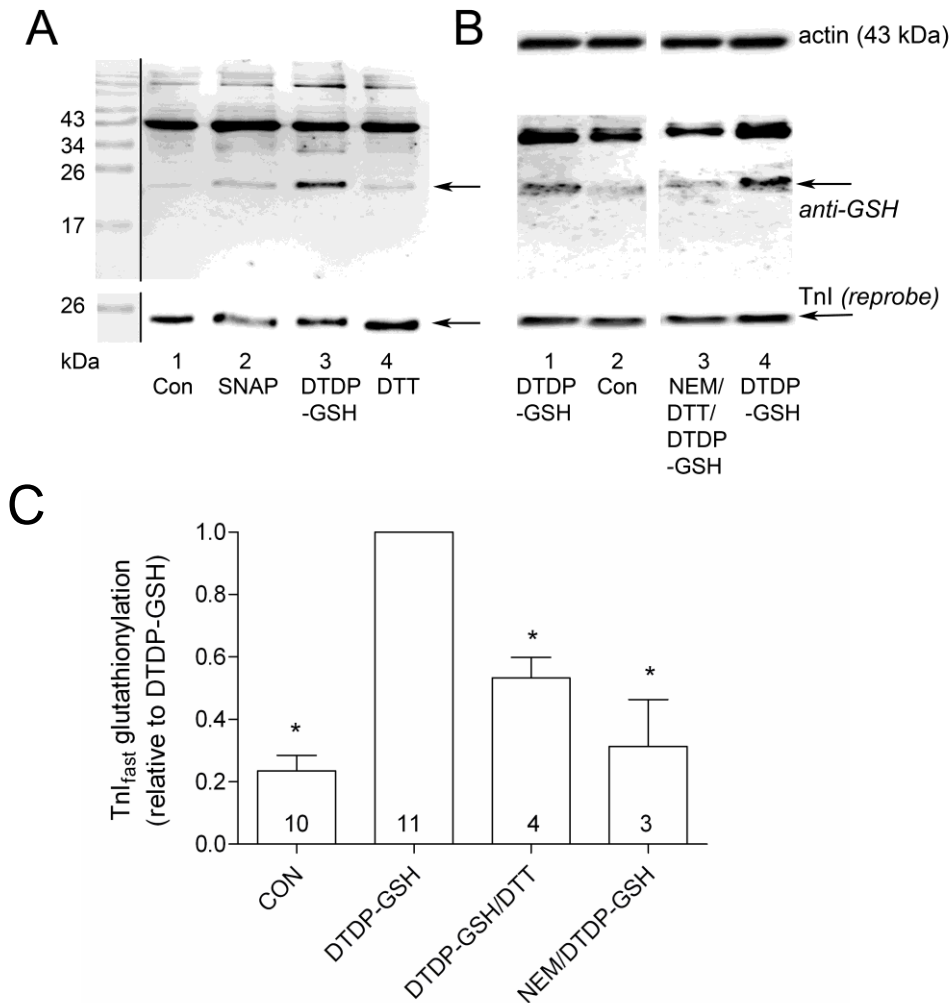


**Figure 2. Constancy of net increase in  $\text{Ca}^{2+}$ -sensitivity with sequential GSSG and DTDP-GSH treatments.** Increase in  $\text{pCa}_{50}$  when individual EDL fibres were first subjected to a submaximal or near maximal GSSG treatment ( $\text{pCa}_{50}$  shift plotted as abscissa value) and then afterwards subjected to the standard DTDP-GSH treatment (additional increase in  $\text{pCa}_{50}$  plotted as ordinate value); the total overall increase in  $\text{pCa}_{50}$  was similar irrespective of the initial GSSG treatment. The different first treatments (all lasting 10 min) were: 10 mM GSSG at pH 7.1 (●); 2.5 mM GSSG at pH 8.5 (▼); 5 mM GSSG at pH 8.5 (◆); and 10 mM GSSG at pH 8.5 (▲). The value on the ordinate axis (■) (i.e. +0.238 pCa units) represents the effect of DTDP-GSH treatment without any first treatment (see Table 1). In all cases subsequent DTT treatment (10 mM, 10 min) fully reversed  $\text{Ca}^{2+}$ -sensitivity to its original level (i.e. by  $\sim -0.24$  pCa units in all cases) (not shown).

**A****B**

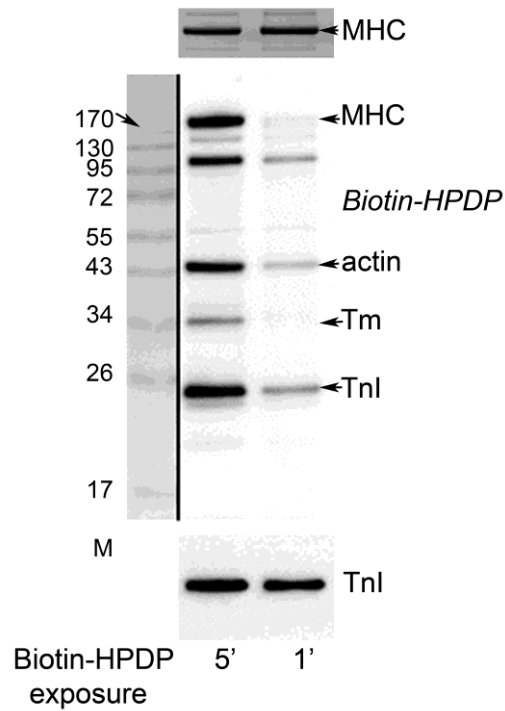
**Figure 3. Pre-treatment with NEM blocks action of DTDP-GSH on  $\text{Ca}^{2+}$ -sensitivity.** **A**, Hill fits for force-pCa data for a rat EDL fibre given the standard DTDP-GSH treatment both before and after treatment with NEM (25  $\mu\text{M}$ , 2 min, at pCa >9). Two successive force-pCa staircases examined after each treatment, with very similar results; data only for second in each pair plotted here. **B**, pCa<sub>50</sub> values following indicated treatment for data in A. Note that there is a small progressive decline in pCa<sub>50</sub> with each force-pCa staircase, which occurs even with no treatment (~0.012 pCa units per staircase pair) (here compare ‘CON’ (control) and subsequent post ‘DTT’ responses).





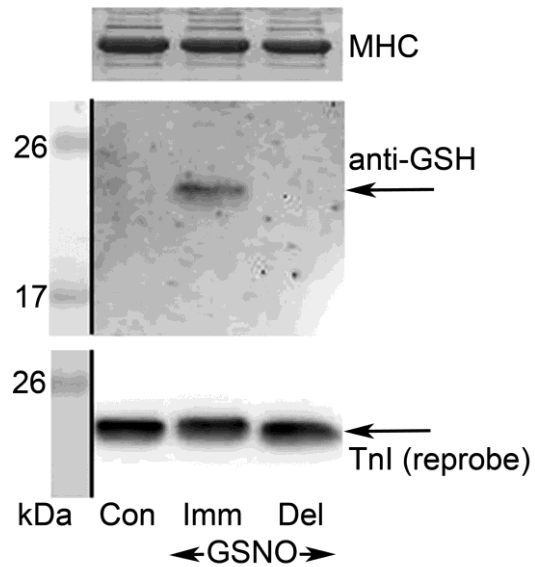
**Figure 4. DTDP-GSH treatment causes S-glutathionylation of troponin I fast isoform (TnI<sub>f</sub>).**

**A**, Upper panel: Western blot with anti-GSH antibody in EDL fibre samples (5 fibre segments per sample) subjected to indicated treatment (all at pCa >9) (15% SDS-PAGE). In all cases fibres washed initially for at least 5 min in pCa > 9. Lane 1, control treatment (Con), 10 min washing at pCa >9; Lane 2, 5 mM SNAP for 5 min; Lane 3, 100  $\mu$ M DTDP for 5 min, 5 mM GSH for 2 min (i.e. ‘standard DTDP-GSH treatment’); Lane 4, 10 mM DTT for 10 min. All samples blocked with 5 mM NEM for 5 min before adding SDS (see Methods). Arrows indicate bands corresponding to TnI. The bands at ~43 kDa correspond to actin. Lower panel: subsequent reprobe of same membrane for troponin I (TnI); EDL fibres contain only the fast isoform, TnI<sub>f</sub>. Positions of molecular weight markers shown on left (see Methods). **B**, Middle panel: anti-GSH blot of EDL fibres given indicated treatments as in panel A. (12.5% SDS-PAGE). Lane 3: Fibres treated with NEM (200  $\mu$ M, 2 min), followed by DTT (10 mM, 10 min), then standard DTDP-GSH treatment. Membrane reprobes for TnI (bottom) and then actin (top). **C**, mean  $\pm$ SE ratio of band density for anti-GSH signal relative to corresponding TnI<sub>f</sub> signal for indicated treatments, expressed relative to DTDP-GSH case on same gel. Number of independent gels shown in each bar. \* significantly different ( $P < 0.001$ ) from DTDP-GSH case.

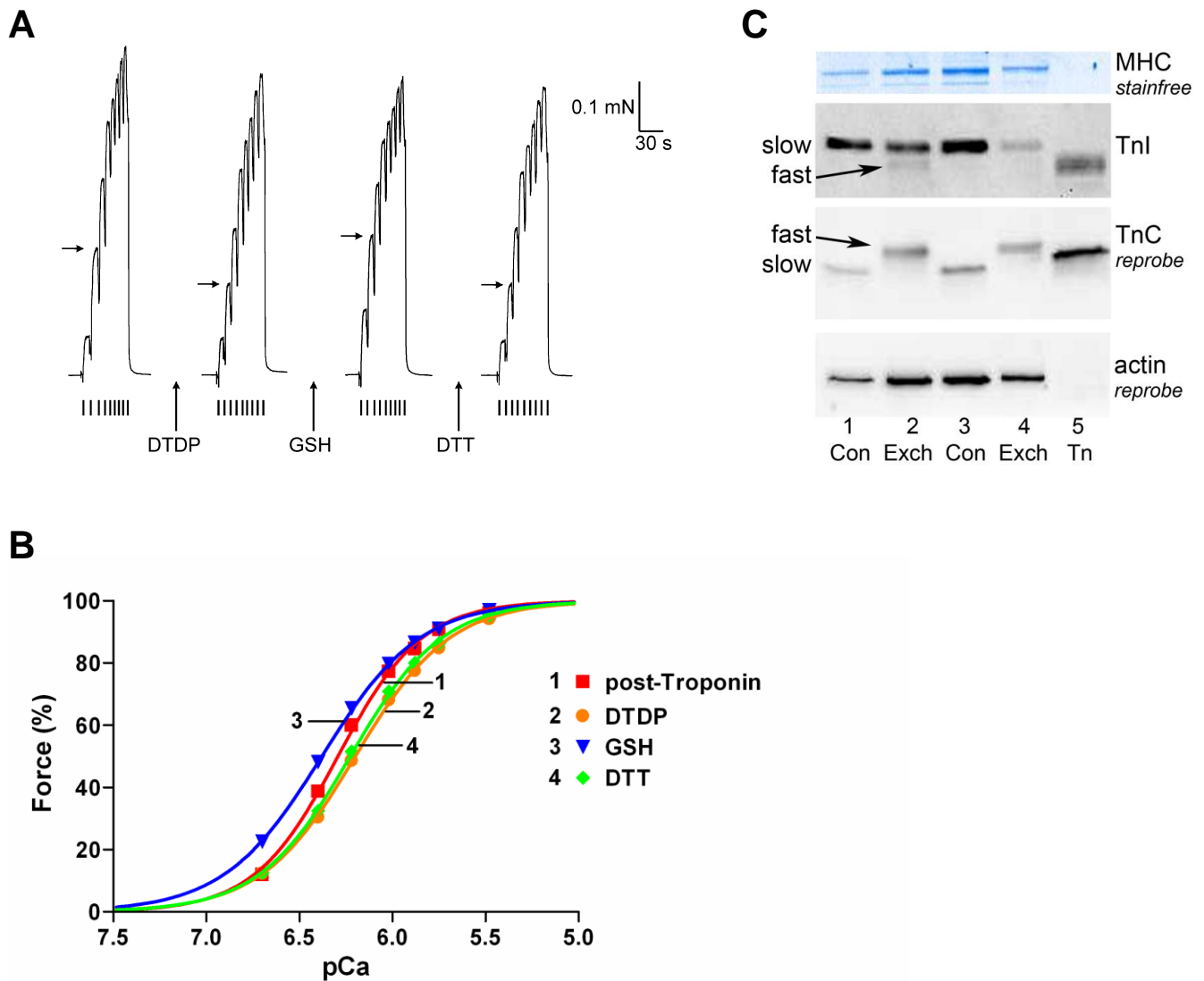


**Figure 5. Biotin labeling of reactive cysteines.**

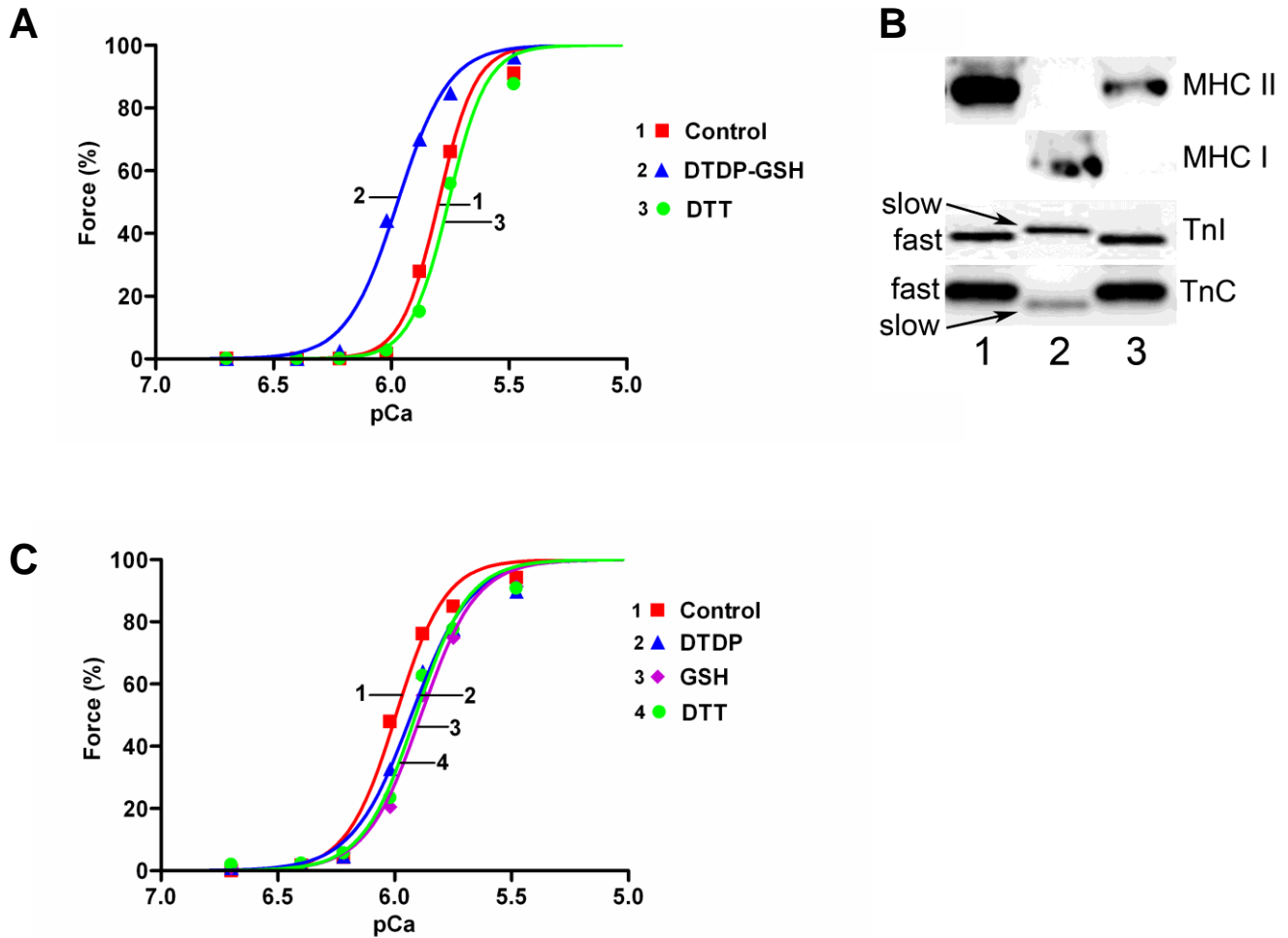
Western blot of single skinned EDL fibre segments treated in relaxing solution with a biotin-tagged thiopyridine reagent (Biotin HPDP) for either 1 or 5 min, and probed for biotin with streptavidin (see Methods). The one min exposure to the reagent produced clear labeling of the TnI isoform present (TnI<sub>f</sub>), but an even a more prolonged (5 min) exposure produced no detectable labeling of either TnC or MLC2 (both run at ~18 kDa). 12.5% SDS-PAGE



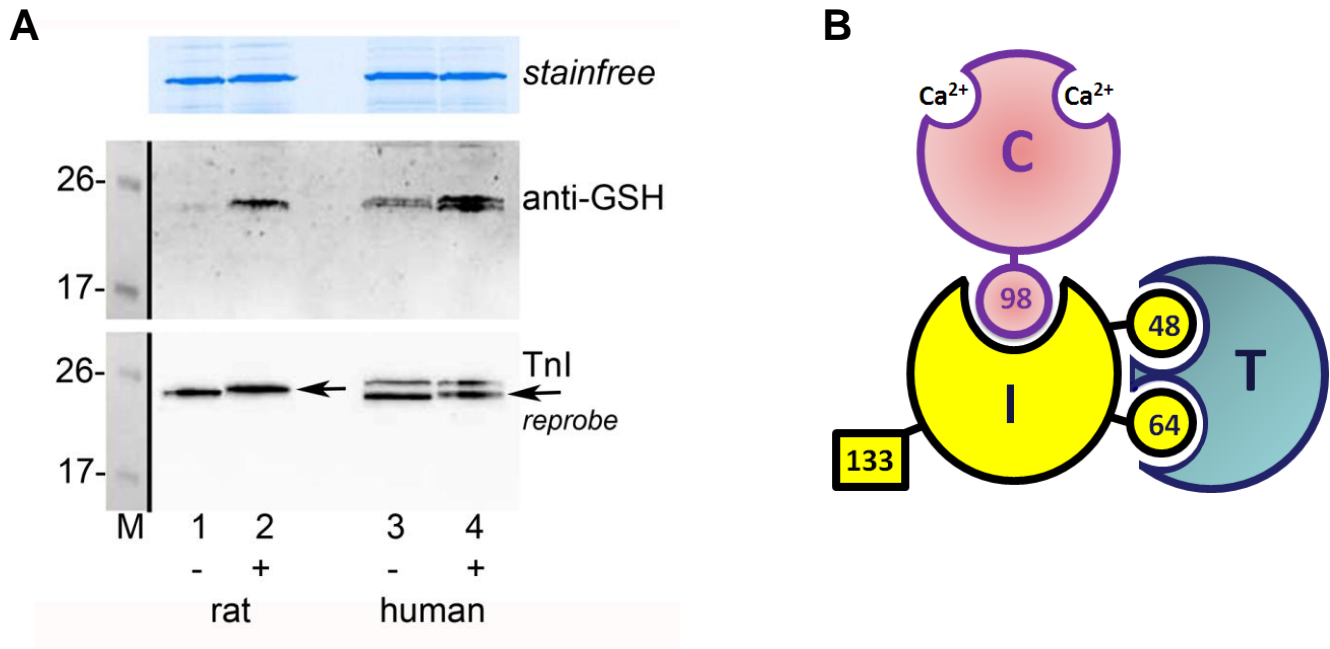
**Figure 6. Freshly-prepared GSNO produces S-glutathionylation of TnI<sub>f</sub>.** EDL type II fibres (4 fibre segments per sample) were subjected to control treatment ('Con', 10 min wash only) or to a 2 min treatment with GSNO (2 mM) either immediately after it was prepared ('Imm') or 10 min after it was prepared (delayed, 'Del'). Fibres first washed for 5 min before either GSNO treatment. All solutions were at pCa >9. Middle panel: Western blot with anti-GSH antibody. Lower panel: Reprobe of membrane for TnI. Top panel: MHC band in commassie-stained gel post-transfer. 15% SDS-PAGE.



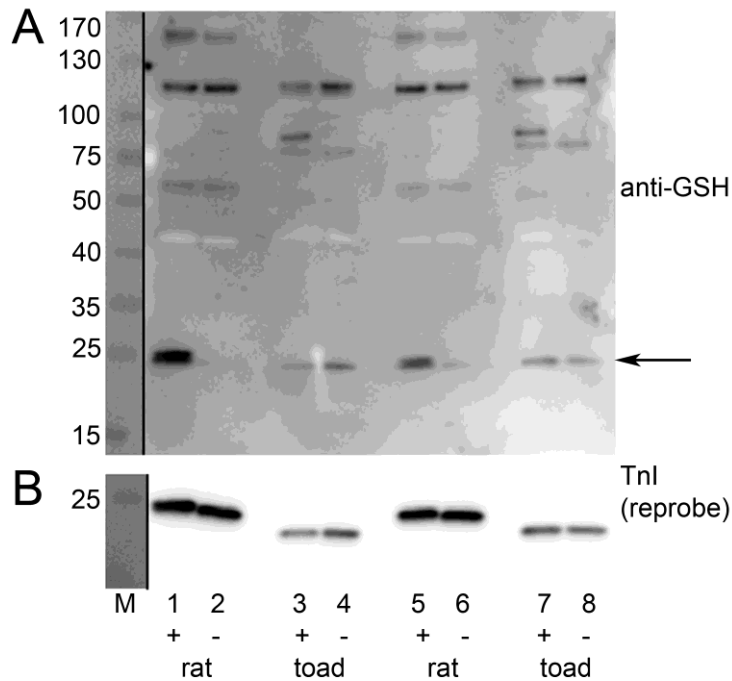
**Figure 7. Effect of S-glutathionylation following partial TnI<sub>f</sub> exchange into a type I fibre.** **A**, Ca<sup>2+</sup>-activated force responses in skinned segment of a rat soleus type I fibre following exchange of porcine fast-twitch troponin (see Methods). [Ca<sup>2+</sup>] initially pCa >9 and raised progressively (small ticks) in order: pCa 6.70, 6.40, 6.22, 6.02, 5.88, 5.75, 5.48, 4.7, >9). Fibre treated successively with DTDP (100 μM, 5 min), GSH (5 mM, 2 min), and DTT (10 mM, 10 min). [Ca<sup>2+</sup>] sequence repeated twice after each treatment, giving very similar force responses; only second of each pair shown. Horizontal arrows indicate force level at pCa 6.40 in each case. **B**, Hill fits to force-pCa data in A. **C**, Western blotting for TnI, TnC and actin in soleus single fibre segments with (Exch) and without (Con) troponin exchange. The segment producing the force responses in A & B was run in Lane 2; virtually all of the endogenous TnC, and ~35% of the TnI, was replaced with the respective fast isoform (see text). Lane 4: another fibre following troponin exchange. Lanes 1 and 3: Untreated control fibres. Lane 5: 0.5 pmol of the exogenous porcine troponin complex (Tn). 8-16% Criterion Stainfree gel.



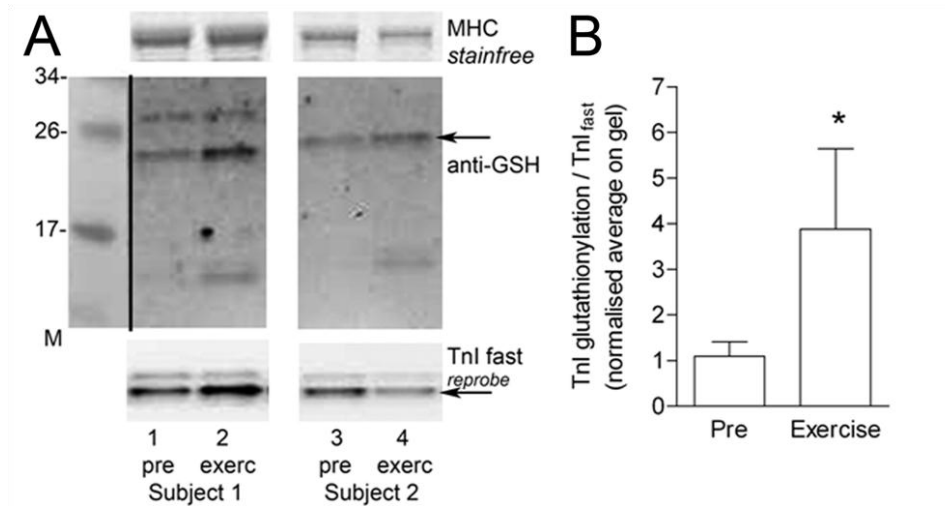
**Figure 8. Glutathionylation treatment causes increased  $\text{Ca}^{2+}$ -sensitivity in human type II fibres but not type I fibres.** Segments of individual fibres from vastus lateralis muscle biopsy from a rested human subject were mounted on the force transducer and force-pCa staircases recorded. **A**, Hill curves for human type II fibre subjected to standard DTDP-GSH treatment and then DTT treatment. **B**, Western blots of human single fibres, probed first for TnI, then TnC, MHCII and finally MHCI. Fibre in panel A run in Lane 1. Force response of fibres to pSr 5.2 in accord with TnC isoform in every case (see text). 4-15% Criterion TGX Stainfree gel. **C**, Hill curves for human type I fibre subjected to successive treatments with DTDP (100  $\mu\text{M}$ , 5 min), GSH (5 mM, 2 min), and finally DTT (10 mM, 10 min).



**Figure 9. TnI<sub>f</sub> runs more slowly when S-glutathionylated.** **A**, Western blots of tissue sections from rat EDL and human vastus lateralis muscles subjected either to a wash only (lanes 1 and 3, ‘-’) or to wash with DTDP (100 μM) and then GSH (5 mM) (lanes 2 and 4, ‘+’). (Adjacent transverse sections of frozen muscles used; treated at room temperature, all in K-HDTA ‘intracellular’ solution at pCa >9). Middle panel: Anti-GSH antibody shows DTDP-GSH treatment increased glutathionylation of TnI<sub>f</sub> in rat EDL muscle and of both TnI<sub>f</sub> and TnI<sub>s</sub> in human muscle (lower and upper bands respectively). Bottom panel: Reprobe with TnI antibody: only fast isoform (TnI<sub>f</sub>) is present in rat EDL muscle, whereas both TnI<sub>f</sub> and TnI<sub>s</sub> are present in the human muscle. Note that TnI<sub>f</sub> runs at a slightly higher molecular weight following treatment with DTDP-GSH in both the rat and human muscle. 8-16% Criterion Stainfree gel. **B**, Diagrammatic representation of exposed and hidden cysteine residues on the troponin complex in mammalian fast-twitch (type II) muscle fibres (modified from Chong & Hodges, 1982; © 1982 The American Society for Biochemistry and Molecular Biology). Letters I, C and T indicate TnI<sub>f</sub>, TnC<sub>f</sub> and TnT<sub>f</sub> subunits, and numbers indicate cysteine residues on TnI<sub>f</sub> and TnC<sub>f</sub> (there are none on TnT<sub>f</sub>). Only Cys133 on TnI<sub>f</sub> is readily accessible to cysteine reagents. TnI<sub>s</sub> (not shown) lacks an equivalent of Cys133, having Cys38, Cys65 and Cys85, with Cys65 being homologous to Cys 64 on TnI<sub>f</sub>.



**Figure 10. No S-glutathionylation of TnI<sub>f</sub> in toad fibres.** Western blot with anti-GSH (upper panel) and subsequent reprobe for TnI (lower panel). Rat EDL fibres in lanes 1 & 2 and 5 & 6, and toad iliofibularis fibres in lanes 3 & 4 and 7 & 8 (four fibre segments in each sample). Fibres in left lane of each pair (i.e. in lanes 1, 3, 5 and 7) given standard DTDP-GSH treatment (+), and fibres in adjacent lanes (2, 4, 6, and 8) washed in same solution without DTDP and GSH (-). In the rat EDL fibres the DTDP-GSH treatment caused a very marked increase in the anti-GSH signal corresponding to TnI<sub>f</sub> (arrow) (compare lanes 1 & 3 with 2 & 6), but no other proteins in the rat fibres showed much increase with treatment. (Note that the anti-GSH signal at ~43 kDa arising from actin is overexposed and appears white). TnI<sub>f</sub> in the toad fibres displayed no anti-GSH signal, either with or without the treatment; note that the anti-GSH signal seen in the toad fibres running similar to the rat TnI<sub>f</sub> signal does not arise from the toad TnI<sub>f</sub>, which runs at an appreciably lower molecular weight, as seen in the TnI reprobe below. M: molecular markers (see Methods). 4-15% Criterion TGX Stainfree gel.



**Figure 11. Increased S-glutathionylation of TnI<sub>f</sub> in human muscle with prolonged exercise.**

**A**, western blot with anti-GSH, and subsequent reprobe for TnI, of transverse sections from vastus lateralis muscle biopsies taken from two human subjects both before ('pre') and after 40 min cycling exercise at ~60% of VO<sub>2</sub>max ('exerc'). The anti-GSH signal indicated by arrow overlaid with the TnI<sub>f</sub> bands (lower of the two TnI bands). Increased glutathionylation was also evident at ~14 kDa in these two post-exercise cases. Top panel shows corresponding MHC bands imaged in stainsfree gel before protein transfer. 4-20% Criterion Stainfree gel. **B**, Mean data (+sem) from 5 subjects for the TnI<sub>f</sub> S-glutathionylation signal before and after exercise (all samples run on same gel, repeated three times). The density of each TnI<sub>f</sub> glutathionylation signal was first normalised by the corresponding TnI<sub>f</sub> band density, and then each value was re-expressed relative to the mean of the pre-exercise cases on that gel (effectively declaring latter as '1'); values from 3 repetitions averaged to yield a single value for each pre- and post-exercise sample for each subject. \* significantly greater than 'pre' (paired t-test, n = 5 subjects, P<0.05, one-sided Wilcoxin signed rank test).



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