Effects of *S*-glutathionylation on the passive force-length relationship in skeletal muscle fibres of rats and humans

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Abstract

This study investigated the effect of *S*-glutathionylation on passive force in skeletal muscle fibres, to determine whether activity-related redox reactions could modulate the passive force properties of muscle. Mechanically-skinned fibres were freshly obtained from human and rat muscle, setting sarcomere length (SL) by laser diffraction. Larger stretches were required to produce passive force in human fibres compared to rat fibres, but there were no fibre-type differences in either species. When fibres were exposed to glutathione disulfide (GSSG; 20 mM, 15 min) whilst stretched (at a SL where passive force reached ~20% of maximal Ca^{2+}-activated force, denoted as $SL_{20\%\ max}$), passive force was subsequently decreased at all SLs in both type I and type II fibres of rat and human (e.g., passive force at $SL_{20\%\ max}$ decreased by 12% to 25%). This decrease was fully reversed by subsequent reducing treatment with dithiothreitol (DTT; 10 mM for 10 min). If freshly skinned fibres were initially treated with DTT, there was an increase in passive force in type II fibres (by 10 ± 3% and 9 ± 2% in rat and human fibres, respectively), but not in type I fibres. These results indicate that *i)* *S*-glutathionylation, presumably in titin, causes a decrease in passive force in skeletal muscle fibres, but the reduction is relatively smaller than that reported in cardiac muscle, *ii)* in rested muscle *in vivo*, there appears to be some level of reversible oxidative modification, probably involving *S*-glutathionylation of titin, in type II fibres, but not in type I fibres.

Introduction

It is well known that reactive oxygen species (ROS) production increases when a muscle actively works (Lamb and Westerblad 2011; Powers and Jackson 2008). *S*-glutathionylation is a ROS-mediated chemical process in which a mixed disulfide is formed by glutathione reacting with an oxidized cysteine or by an oxidized glutathione reacting with a reduced cysteine (Dalle-Donne et al. 2007). In particular proteins, *S*-glutathionylation can cause a substantial change in their functional properties. For example, *S*-glutathionylation of the fast isoform of troponin I in mammalian skeletal muscle results in a large increase in the Ca^{2+} sensitivity of the contractile apparatus (Dutka et al. 2017; Mollica et al. 2012). Similarly, titin, also known as connectin, becomes markedly more elastic in cardiac muscle if the 'cryptic' cysteines in its Ig domains, which are normally hidden but exposed by stretch, become *S*-glutathionylated (Alegre-Cebollada et al. 2014). Cardiac muscle contains the N2BA and the N2B isoforms of titin, whereas skeletal muscle contains the N2A isoform, and a number of properties of titin, in particular the passive force-length relationship, are quite different between the cardiac and skeletal isoforms. It is currently unknown whether *S*-glutathionylation causes any change in the passive force properties of skeletal muscle, though any such effect would likely have appreciable functional consequences.

Titin is an elastic filamentous protein and the main determinant of passive force in muscle (Maruyama et al. 1976; Wang et al. 1979). Its NH_2 terminus is in the Z-disk and reaches all the way to the center of the sarcomere, i.e., to the M-line. The I-band segment of titin, starting from ~100 nm away from the center of Z-disk, acts as a molecular spring and consists of immunoglobulin-like (Ig) domain segments interspaced with N2A and PEVK domains in the skeletal muscle phenotype (Linke 2018). When the titin is stretched from the slack condition, the I-band segments are first straightened and no passive force is produced. Further

stretching leads to extension of the flexible PEVK domain and unravelling of individual Ig domains, and passive force increases approximately exponentially (Hsin et al. 2011; Kruger and Linke 2011; Linke et al. 1996; Wang et al. 1991).

The contractile characteristics of a muscle fibre are predominantly determined by the myosin heavy chain (MHC) isoform expressed, but can also be affected by other structural proteins, such as titin (Prado et al. 2005; Rivas-Pardo et al. 2016), as well as by intracellular conditions (Allen et al. 2008). Previous studies have shown that the intracellular components, e.g., sarcoplasmic reticulum and myofibrillar proteins, can be more oxidized *in vivo* in type II fibres than type I fibres (Lamboley et al. 2015; Lamboley et al. 2016), possibly owing to the lower antioxidant activity in type II fibres compared to type I fibres (Higuchi et al. 1985; Ji et al. 1992; Powers and Jackson 2008). Since *S*-glutathionylation of intracellular proteins is seen in resting muscle fibres (Mollica et al. 2012), it is possible that the level of *S*-glutathionylation of titin is different *in vivo* between type I and type II fibres, which may differentially affect passive force production in the two fibre types. However, this point has not been investigated to date.

The purpose of this study was to examine the effects of *S*-glutathionylation on elasticity in skeletal muscle fibres. Passive force in skeletal muscle is determined primarily by the properties of both titin and extracellular collagen (Linke and Kruger 2010; Prado et al. 2005). Here, we used mechanically-skinned skeletal muscle fibres in which the passive force is dictated almost exclusively by titin elasticity because the sarcolemma and basement membrane (including collagen and laminin) are absent. Both type I and type II fibres from human and rat skeletal muscle were examined to investigate whether there is any difference in the properties of passive force between the fibre types.

Materials and Methods

Ethical approvals

All rat protocols and procedures were approved by the La Trobe University Animal Ethics Committee and by the Animal Care Committee of Hiroshima University. Sprague-Dawley rats (7 in total, male, 7 to 8 months old) were housed at controlled temperature (22°C) and a 12:12 h light-dark cycle, with food and water provided *ad libitum*. The rats were killed by overdose of isoflurane (4 % vol/vol) in a glass chamber in the experiments at La Trobe University and by overdose of pentobarbital sodium (200 mg/kg body weight) in the experiments at Hiroshima University, and then the extensor digitorum longus (EDL) and soleus muscles were removed by dissection. Subsequently, the muscles were placed in room temperature paraffin oil (Ajax Chemicals, Sydney, Australia) and gradually cooled on ice to ~10 ºC. Note that the passive force properties and the effects of *S*-glutathionylation were very similar in the experiments at both laboratories at La Trobe and Hiroshima University.

For the human experiments, all protocols and procedures were approved by the Human Research Ethics Committees at Victoria University and at 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. All experiments on human skinned fibres were performed on fibres obtained from *vastus lateralis* muscle biopsies from 7 rested subjects (Male, 20 to 32 years old, 75.1 ± 3.4 kg body weight). All subjects were healthy and most participated in regular physical activity but were not specifically trained in any sport. After injection of a local anaesthetic (1% lidocaine (lignocaine)) 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. 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.

Skinned fibre solutions

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless specified otherwise. Relaxing solution contained (in mM) 50 EGTA, 8 total ATP, 36 Na^+, 126 K^+, 10.3 total Mg^{2+} (giving 1 mM free [Mg^{2+}]), 10 phosphocreatine (CP), 90 HEPES, pH 7.1, and pCa ($-\log_{10}[Ca^{2+}]$) >9. Maximum Ca^{2+}-activated solution had a similar composition but with all EGTA replaced by CaEGTA (at pCa 4.7) and total magnesium of 8.1 mM to maintain free Mg^{2+} at 1 mM (see ref (Lamboley et al. 2013; Stephenson and Williams 1981)). Dithiothreitol (DTT) was added to relaxing solution at 10 mM final concentration from a 1 M stock made in double-distilled water. A 200 mM stock of oxidized glutathione (GSSG) was made in the relaxing solution; the pH of the stock was re-adjusted either to 7.1 or to 8.5 with KOH, and then diluted 10-fold in the final solution (i.e., relaxing solution at required pH).

Preparations and force recording

Muscles were pinned at resting length in a petri dish lined with Sylgard 184 (Dow Corning, Midland, MI) and immersed in paraffin oil and kept cool (~10°C) on an icepack. Using jeweler's forceps, a section of an individual fibre was mechanically skinned and a segment ~2 mm in length was mounted on a force transducer (AME801, SensoNor, Horten, Norway), initially at 120% of its resting length (see detailed description in (Lamb and Stephenson 2018)). The fibre was then transferred to a 2-ml Perspex bath containing relaxing solution, which broadly mimicked the intracellular milieu. Each fibre was subsequently exposed to a strontium ion (Sr^{2+})-containing solution at pSr ($-\log_{10}[Sr^{2+}]$) 5.2 in order to give

an initial indication of the fibre type, and then maximal Ca^{2+}-activated force was determined by exposure to maximum Ca^{2+}-activation solution. Sr^{2+} directly activates the contractile apparatus but the sensitivity to Sr^{2+} differs markedly between type I and type II fibres. Fibres containing the slow-twitch isoform of troponin C (TnC) give close to the maximum Ca^{2+}-activated force level at pSr 5.2, whereas fibres containing the fast twitch isoforms of TnC produce <5% of maximum force, and fibres with a mixture of the fast and slow isoform of TnC produce an intermediate level of force (Bortolotto et al. 2000; Lamboley et al. 2013; O'Connell et al. 2004). The fibre type indicated by the Sr^{2+} response matched that found by subsequent western blotting (see later) in every human fibre examined in the present study (e.g. Fig. 1). The maximum Ca^{2+}-activated force level was also used to gauge the subsequent passive-force measurements (see 'Elasticity test and treatments').

Sarcomere length measurement

Sarcomere length (SL) was determined by the diffraction pattern produced by a He-Ne laser beam passing through the skinned fibre preparation, as described previously (Stephenson and Williams 1981). The average sarcomere length SL was calculated from the position of the first-order diffraction maxima using the approximate expression (Eq. 1) which included a correction factor for the passage of the diffracted laser beam through the solution around the preparation and through the wall of the spectrophotometric vial before propagation though the air:

$$SL = WL \times \left[1 + \frac{4 \times (d_{air} + n_w \times d_w + n_v \times d_v)^2}{{d_{11}}^2}\right]^{0.5} \qquad (1)$$

where WL is the laser wave-length (0.670 μm), and d_w (3-5 mm), d_v (1.0 mm), and d_{air} (200 mm), the distances travelled by the zero-order diffracted beam through the solution, the polystyrene wall of the vial, and air before reaching the screen, respectively; n_w (1.3) and n_v

(1.5) are the refractive indices of water and polystyrene, respectively, and d_{11} the distance (in mm) between the centers of the first-order diffraction maxima measured on the screen.

Elasticity test and treatments

All force measurements were made at room temperature (23 ± 2 °C). Each skinned fibre segment was placed into the modified polystyrene spectrophotometric vial containing relaxing solution. The fibre was then slacked off and subsequently stretched again until producing just detectable force, and this was defined as the resting length. Thereafter, the passive force-length relationship was measured: the fibre was stretched from resting length to various longer SL until the passive force reached the equivalent of ~20% of the maximal Ca^{2+}-activated force measured (this SL was denoted as '$SL_{20\%\ max}$'). The diffraction maxima points at each SL in this first series of passive force measurements on the given fibre were recorded on the screen, so that the fibre could be readily stretched again to the same SLs after subsequent treatments and procedures.

After the measurements of passive force on the fibre in its initial state, the fibre was subjected to each of following treatments (except in the experiments indicated) in the order: 10 mM DTT for 10 min, 20 mM GSSG for 15 min, then 10 mM DTT for 10 min. Each treatment was applied in relaxing solution with the fibre stretched to the $SL_{20\%\ max}$ determined on the initial measurements (except in the experiments noted where the GSSG treatment was applied with the fibre at resting length). Then the fibre was returned to relaxing solution and readjusted back to resting length, and then the passive force properties re-examined. In most experiments, the GSSG treatment solution was at the standard pH level of 7.1, but in an additional set of cases the fibre was treated with GSSG at pH 8.5. In the latter cases, the effect on the passive

force properties of exposure to relaxing solution at pH 8.5 for 15 min without any GSSG was first determined before examining the effect of GSSG at pH 8.5. At the end of the measurements, each human fibre was kept for subsequent western blotting in order to ascertain the myosin heavy chain isoforms present (see 'Western blotting').

Passive force was defined as the steady-state force level attained after stretching the fibre to the given SL, or in the case of long SLs, as the force level reached 2 min after the stretch (see Fig. 2). For each fibre, the passive force values were fitted with the following exponential function:

$$F(SL) = A \times \exp\left((SL - SL_e)/\lambda\right) \qquad (2)$$

where F(SL) is the passive force at the given SL, A is a scaling constant, SL_e is the SL at which there is just detectable passive force, and λ is a length constant. A and λ were determined by best curve fitting with GraphPad Prism version 6.

Western blotting

After force measurements, each human fibre was placed in a small volume (15 µl) of solubilizing buffer containing 0.125 M Tris-HCl, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol and ~0.001% bromophenol blue (pH 6.8) diluted (2:1 vol./vol.) with double distilled water. Fibres were stored at −80°C until analysis. Total protein in the single fibres was separated on 4–15% Criterion Stain Free gels (Bio-Rad, Hercules, CA, USA) and wet-transferred to nitrocellulose for 30 min at 100 V in a circulating ice-cooled bath with transfer buffer containing 25 mM Tris and 192 mM glycine at pH 8.3 and 20% methanol. After appropriate washes and blocking (5% skimmed milk in Tris-buffered saline with Tween, 1-2 h), membranes were probed with primary antibodies and appropriate secondary antibodies. The

following primary antibodies were used in the order: MHCIIx (mouse IgM, DSHB, cat. no. 6H1, 1 in 100), myosin heavy chain II (MHCIIa, mouse IgG, Development Studies Hybridoma Bank (DSHB), cat. No. A4.74, 1 in 400) and MHCI (mouse IgM, DSHB, cat. No. A4.840, 1 in 100). The secondary antibodies were: goat anti-mouse IgG (1 in 20,000; Thermo Fisher Scientific, cat. no. PIE31430) and anti-mouse IgM (1 in 60,000; Santa Cruz Biotechnology Dallas, Texas, USA, cat. no. sc-2064). Chemiluminescent images were captured using the Chemidoc MP (BioRad) following exposure to West Femto chemiluminescent reagent (Thermo Fisher Scientific) and densitometry performed using ImageLab software (BioRad). The MHCIIx, MHCIIa and MHCI images were obtained in the same membrane with stripping between each imaging (e.g., Fig. 1).

Statistics

Values are expressed as mean ±SE. The normality of the data was first tested by the Shapiro-Wilk normality test. Student's two-tailed t-test or Wilcoxon single rank test was used for data sets showing normal or non-normal distributions, respectively. Pearson correlation coefficient analysis was used to evaluate whether there was a linear relationship between the size of the GSSG effect on passive force and λ for the data in the Fig. 5B. For each passive force-length relationship, the goodness of fit of the exponential function was evaluated by the R^2 value.

Results

Effect of dithiothreitol on the passive force-length relationship in freshly skinned fibres

After first examining the passive force properties in each skinned fibre in its initial state (e.g. leftmost trace in Fig. 2A), each fibre was exposed to 10 mM DTT for 10 min whilst stretched at $SL_{20\%\ max}$ in order to test whether this strong reducing treatment altered the passive force properties (see Fig. 2). In both human and rat fibres, the passive force-length relationship became stiffer after such DTT treatment in type II fibres, but not in type I fibres (see Fig. 3: passive force at $SL_{20\%\ max}$ in type II fibres was increased by 13.4 ± 3.5% and 9.3 ± 2.4% in rat and human, respectively). Subsequent western blotting demonstrated that all the human type II fibres examined in this particular experiment were pure IIa fibres.

Effect of S-glutathionylation on the passive force-length relationship

To examine the effect of *S*-glutathionylation on passive force in skeletal muscle, following the initial DTT treatment, each skinned fibre was treated (whilst again stretched) with 20 mM GSSG at pH 7.1 for 15 min. As a result, the passive force was subsequently decreased at all SL measured (e.g., Fig. 2), with the mean decrease measured at $SL_{20\%\ max}$ being 25.7 ± 3.5%, 12.0 ± 4.0%, 15.1 ± 4.3% and 14.1 ± 4.0% in rat type II, rat type I, human type II and human type I, respectively (Fig. 3). This effect of GSSG was fully reversed by re-application of the DTT treatment (e.g., Fig. 2). In the subset of fibres examined, it was further found that the passive force was not altered if the GSSG was applied when the fibre was not stretched (e.g. Fig.s 2 and 3). The effect of 20 mM GSSG treatment at pH 8.5 was also examined in some rat fibres, because most cytoplasmic proteins contain cysteine sulfhydryls that have a pKa value greater than 8.0, and the higher pH should decrease their protonation and greatly increase their reactivity to GSSG (Dalle-Donne et al. 2007; Mollica et al. 2012). It was found that the effect on passive force of GSSG treatment at pH 8.5 was not noticeably different

from that at pH 7.1 in either type I or type II fibres (decreases of 23.8 ± 1.5% (n=3) versus 24.9 ± 4.8% at $SL_{20\%\ max}$ at pH 8.5 and 7.1, respectively, in type II fibres, and decreases of 14.7 ± 6.2 (n=3) versus 12.0 ± 4.0%, respectively, in type I fibres). These results suggest that the GSSG treatment at pH 7.1 was sufficient to elicit the maximal possible level of *S*-glutathionylation for the conditions examined.

Additionally, to examine whether the effect of GSSG on passive force resulted purely from the initial DTT treatment cleaving normal intra-molecular disulphide bonds present *in situ*, in further experiments the GSSG treatment was applied to freshly skinned EDL fibres (i.e., with no pre-treatment with DTT). In these cases the passive force at $SL_{20\%max}$ was still decreased by the GSSG treatment (by 28.2 ± 2.2% of control (n = 3); mean value expressed taking into account the change upon subsequently reversing the GSSG effect with DTT treatment); this is comparable with the size of the decrease found above when the GSSG was applied after an initial DTT pre-treatment (25.7 ± 3.5%, n=9). Thus, the effect of the GSSG treatment in decreasing passive force was not simply an artefact caused by the initial DTT treatment breaking normal -S-S- crossing links.

In each fibre, the force-length relationship found in each treatment state could be well fitted by an exponential function, starting at a SL defined as SL_e, and using two variables: a length constant, λ, and a scaling factor, *A* (see Material and Methods) (e.g., Fig. 2B). Following GSSG treatment, the best fit curves had decreased *A* and/or increased λ, both changes indicating that the force-length relationship became less steep (Fig. 2). However, the change in each parameter was not consistent between different fibres: there was a decrease in *A* with little change in λ in some fibres, whereas there was an increase in λ with little change in *A* in other fibres. This likely reflects the fact that many factors (e.g., Ig domains and PEVK domain)

279 contribute to the increased passive force with extension, and that the data are modelled better
280 by more complicated functions (e.g., freely jointed chain model or worm-like chain model: see
281 ref (Hsin et al. 2011)) rather than a simple exponential fit. Consequently, the presentation and
282 statistical analysis of the data on the changes occurring with DTT and GSSG treatment are
283 based on the percentage change in passive force at $SL_{20\% max}$ (Fig. 3) rather than on the changes
284 in the exponential fit parameters.

285

286 *Difference of passive force-length relationship between type I and type II fibres*

287 The passive force-length relationship can differ substantially between different fibre
288 types and different muscles in some animals (Prado et al. 2005), although a previous study on
289 human *vastus lateralis* muscle fibres observed little or no fibre-type differences (Olsson et al.
290 2006). To further investigate this point, the passive force-length relationship was compared
291 between type I and type II fibres in both rat and human. Data from all fibres examined are
292 presented in Fig. 4 and show that there was no consistent difference in force-length relationship
293 between the type I and type II fibres in either rat or human. Moreover, there was no significant
294 difference in the exponential fit parameters of the force-length relationships between the type
295 I and type II fibres in either species (Table 1). It was apparent, however, that there was
296 substantial heterogeneity in the passive properties between the different human type II fibres
297 examined (see blue curves in Fig. 4B). Of these thirteen fibres, ten were pure type IIa and three
298 were type IIa/x, and the greatest disparity in the curves occurred between different pure IIa
299 fibres. The best fit parameters to the passive force data in the IIa/x fibres did show a
300 significantly lower A and lower λ values than in the type IIa fibres (A: 0.24 ± 0.09 in type IIa/x
301 versus 0.70 ± 0.14 in type IIa; λ: 0.38 ± 0.04 in type IIa/x versus 0.50 ± 0.04 in type IIa), but
302 these differences in the two fit parameters have opposing effect on the steepness of the fit
303 function, indicating subtle but complex differences in the exact shape of the fits (see previous

304 section). Furthermore, these three IIa/x fibres were all obtained from the same subject and the
305 fit parameters to the passive force in the one type IIa fibre examined from the same subject also
306 showed similar low *A* (0.15) and λ (0.33) values. Thus, it seems that the differences in the fit
307 parameters observed between the type IIa/x and type IIa fibres was more likely due to a subject-
308 to-subject difference rather than to a true fibre-type difference (see also following section).

309

310 It is also apparent in Fig. 4 that there were significant differences in the passive force
311 properties between rat EDL and soleus fibres and human *vastus lateralis* fibres. The fit
312 parameters in human fibres had a higher mean SL_e and a higher λ value (Table 1), indicating
313 that the passive force on average started to increase at a longer SL, and increased less steeply
314 with stretching, in the human *vastus lateralis* fibres compared to the rat EDL and soleus fibres.

315

316 *Passive force-length relationship in human type II fibres: difference between subjects*

317 As mentioned, the passive force-length relationship was more heterogeneous in the
318 human type II fibres than in the human type I fibres or in rat fibres (Fig. 4). Fig. 5A shows the
319 passive force-length relationships in the human type II fibres sorted by subject; these data
320 appear to indicate that the heterogeneity in the properties may have been subject dependent. It
321 is also interesting to note that correlation analysis of the passive force changes occurring with
322 GSSG treatment in human type II fibres (Fig. 5B) indicated a significant inverse relationship
323 between the size of the best fit length constant (λ) for a given fibre and the extent of the decrease
324 in passive force with GSSG treatment in that fibre ($r = -0.909$, $P = 0.033$). In other words, the
325 type II fibres showing greater elasticity (i.e., larger λ) underwent a larger relative decline in
326 passive force with *S*-glutathionylation treatment; however it is noted that this analysis was
327 based on examination of a total of only 5 type II fibres from three subjects.

328

329 *Effect of overstretch on the passive force*

330 As observed previously (Linke et al. 1996; Wang et al. 1991), it was found that with
331 very large stretches the passive force-length relationship in fibres eventually started to level off
332 from its initial exponentially increasing phase (e.g. Fig. 6A), possibly due to the dislodgment
333 of the titin-thick-filament anchorage. We defined SL_y, the SL at which the passive force starts
334 to yield, as the SL where the initial exponential curve intersects with a line joining the higher
335 SL force data (see Fig. 6B). This yield point occurred at significantly shorter SL in rat type II
336 fibres than in human type II fibres (mean SL_y: 3.88 ± 0.04 and 4.62 ± 0.08 μm, respectively, P
337 < 0.05, Fig. 6C). In addition, it was observed that the passive force at a given SL was markedly
338 decreased after extreme stretch beyond SL_y. Specifically, in rat type II fibres, the passive force
339 at a SL of ~3.4 μm decreased to 44.1 ± 2.3% of the initial level (n=4) after extreme stretch at
340 ~4.3 μm SL. In contrast, when fibres were stretched only to SL less than SL_y, the passive force
341 properties remained unchanged.

342

Discussion

Effects of S-glutathionylation of passive force in skeletal muscle

We investigated the effect of *S*-glutathionylation on the elasticity of titin in skeletal muscle using mechanically-skinned fibres. The present results indicate that *i)* *S*-glutathionylation, presumably in titin, causes a reversible decrease in passive force in skeletal muscle fibres (Fig.s 2 and 3), and *ii)* in rested muscle *in vivo*, titin is already reversibly oxidized to some extent, probably via *S*-glutathionylation, in type II fibres but not in type I fibres (Fig.s 2 and 3).

Previous studies have shown that passive force production in skeletal muscle is due primarily to the effects of both titin and extracellular collagen (Linke 2018; Linke and Kruger 2010). The skinned fibre was used here as a tool to examine the elasticity of titin, because the sarcolemma and the basement membrane could be straightforwardly removed (Olsson et al. 2006; Wang et al. 1991). It was found here that *i)* passive force was subsequently decreased after a skinned fibre had been exposed to GSSG whilst stretched but not if it was exposed to GSSG whilst at resting length, and *ii)* the decreased passive force was restored by subsequent reducing treatment with DTT. Alegre-Cebollada et al. (2014) have previously shown that GSSG treatment similar to that used here results in *S*-glutathionylation of titin in cardiac muscle and a decrease in passive force, but only if the GSSG is applied whilst the muscle is stretched. In view of this similarity, we believe it is most likely that the GSSG-induced decrease in passive force seen here is also due to *S*-glutathionylation of titin. As we did not obtain any direct chemical evidence of *S*-glutathionylation of titin, it is possible that the effect was instead due to *S*-glutathionylation of some other cytoskeletal proteins (e.g., β-tubulin). However, this seems doubtful given that passive force in skinned fibres is due to stretching of titin, and the GSSG

was only able to exert its effect on passive force when the fibre was stretched (Fig. 2).

In cardiac muscle *S*-glutathionylation of titin results in a very marked decrease in passive force, to ~15% of the control value, whereas in the present study comparable *S*-glutathionylation treatment in skeletal muscle resulted in a substantially smaller decrease in passive force, e.g., decrease to ~75% of the control level in rat type II fibres (Fig. 3). In the present study, the *S*-glutathionylation treatment was applied with the fibre only stretched to a SL where the steady-state passive force was ~20% of the maximum Ca^{2+}-activated force level (i.e., ~4.2 μm SL, in the case of human fibres; Fig. 4) because the titin-thick filament anchorage can become dislodged if the stretching is much greater than this (e.g., >~4.6 μm SL in the case of human fibres; Fig. 6C). It is expected that the stretch used should have been large enough to reveal some Ig domains in skeletal muscle fibres because recent studies have indicated that individual Ig domains in human *vastus lateralis* muscle fibres become unfolded with stretch in the physiological range, i.e., to ~3.0 - 3.4 μm SL (Linke 2018; Linke and Kruger 2010; Rivas-Pardo et al. 2016). It is unclear why *S*-glutathionylation resulted in a much smaller decrease in passive force in skeletal muscle than in cardiac muscle. It is possible that fewer cryptic cysteines were revealed by the stretch in the skeletal titin than in the cardiac titin, or alternatively that the critical cryptic cysteines are less reactive in the skeletal isoform. Alegre-Cebollada et al. (2014) observed that the kinetics of *S*-glutathionylation could be different between cysteines even in the same Ig domain, probably because the propensity for *S*-glutathionylation was decreased by the presence of an adjacent negatively-charged residue.

There were substantial differences in elasticity across the sample of human type II fibres examined here (Fig. 4B) and this difference seems not to be explained by different fibre subtype, because the large variation was observed even in pure type IIa fibres (see Results).

The apparent subject-dependence in the spread of the data (Fig. 5A) raises the possibility that the fibre properties differed between different subjects. Such differences could be the result of splice variants of titin, given that a high level of different alternative splicing events of the titin N2A isoform have been seen in skeletal muscle of different subjects. Furthermore, most of those splice variant occurred in the I-band region, which largely determines the passive force-length relationship and is the target region of *S*-glutathionylation (Linke 2018; Savarese et al. 2018). Interestingly, the elastic properties were found to be far more homogeneous in the rat fibres (Fig. 4A), and it is expected there would be far less variability of titin splicing in rats owing to their in-bred status.

It was also found that the human type II fibres showing greater elasticity (i.e. a larger λ value for the length-force relationship) underwent a greater decrease in passive force with GSSG treatment (see Fig. 5B). The larger λ value indicates that these fibres were held at a greater SL during the GSSG treatment (because they had to be stretched to such longer SL to reach the specified passive force level, i.e. 20% of maximum Ca^{2+}-activated force). Thus, it is possible that this greater stretch revealed more cryptic cysteine residues on the titin, allowing the GSSG treatment to elicit a larger decrease in passive force.

Differences in the resting oxidative state between fibre types

In the freshly skinned fibres used here from both rat and human muscle, treatment with the reducing agent DTT resulted in an increase in stiffness in the type II fibres but not in the type I fibres (Fig. 3). This effect of DTT indicates that the titin in the type II fibres was oxidized to some extent *in vivo*, most likely by *S*-glutathionylation, although it cannot be ruled out that the lower stiffness was instead the result of some other type of DTT-reversible oxidative modification of the titin. DTT is only able to reduce disulfide bonds, including

417 *S*-glutathionylation (-SSG), as well as *S*-nitrosylation (-SNO) (Dutka et al. 2017; Jaffrey and
418 Snyder 2001) and sulfenation (-SOH) (Saurin et al. 2004), and is not able to reduce sulphinic
419 ($-SO_2H$) and sulphonic ($-SO_3H$) products (Cleland 1964; Halliwell and Gutteridge 2015) nor
420 reverse lipid adduction (-S-lipid) (Dogterom et al. 1989). It has been shown in cardiac titin that
421 formation of an intra-protein disulfide bridge induces an *increase* in the stiffness of titin, which
422 can be reversed by DTT (Beckendorf and Linke 2015; Grutzner et al. 2009). Given that DTT
423 treatment had the opposite effect on stiffness in the skeletal muscle fibres here (i.e. increase
424 not decrease), it seems unlikely that the lowered stiffness seen in the freshly-skinned type II
425 fibres was due to the presence an intra-protein disulfide bridge like the one that can be induced
426 in cardiac muscle titin, particularly given that the effect in cardiac titin occurs in the N2-Bus
427 region, which is specific to cardiac titin and not present in skeletal titin.

428

429 Antioxidant enzyme activity (e.g., superoxide dismutase activity, and total glutathione
430 level) is higher in type I than in type II muscle fibres (e.g., ~5-fold higher gluthathione content
431 in type I fibres) (Higuchi et al. 1985; Ji et al. 1992). Such higher antioxidant activity in type I
432 fibres would be expected to help keep the intracellular environment *in vivo* in a more reduced
433 state, which possibly accounts for why the DTT treatment of freshly skinned fibres had a
434 significant effect on the titin stiffness only in the type II fibres and not in the type I fibres
435 examined here (Fig. 3). It is possible also, at least in the case of the rat fibres, that the difference
436 in responsiveness to DTT between the fibre types was the result of the stretches experienced
437 by the fibres *in vivo* with normal daily activity, which might have differed for the type I fibres
438 obtained from soleus muscle compared to the type II fibres from EDL muscle. If the titin had
439 been stretched more in the type II fibres, a relatively larger number of Ig domains of titin would
440 have been unfolded, which could have allowed greater *S*-glutathionylation of the titin. This
441 type of mechanism, however, would not readily explain the difference in the effect of DTT seen

between the type I and type II fibres from human *vastus lateralis* muscle.

Differences in passive force-length relationship between fibre types and species

The passive force-length relationship was not noticeably different between type I and type II fibres in the muscles examined here, in either rat or human (Fig. 4). The human data are in agreement with those found previously in human *vastus lateralis* fibres (Olsson et al. 2006), and are in accord with the observation that all the titin in such muscle appears to be of similar size (~3680 kDa) (Olsson et al. 2006). Likewise, the similarity of the passive force-length relationship in the rat type I and type II fibres, obtained from soleus and EDL muscles respectively, is in accord with the similarity in the size of the titin molecules found in the two muscles (3521 kDa and 3505 kDa, respectively) (Li et al. 2012). Furthermore, the fact that the titin isoform in human *vastus lateralis* muscle is seemingly longer than that in the rat muscles might help account for why passive force only started to rise at longer SL, and was less steep, in human fibres compared with the rat fibres (see Fig. 4 and Table 1). However, this last conclusion should be seen as open to some doubt, because the measurements of titin size in the human and rat muscles were made in different studies (Li et al. 2012; Olsson et al. 2006), and so cannot be directly compared with certainty. Irrespective of the whether the differences are due to differences in titin alone, the findings here clearly demonstrate that the passive force-length relationship is substantially different between human and rat muscle fibres.

Characteristics of passive force with overstretching

When a single fibre is progressively stretched, the passive force initially increases approximately exponentially, but with stretches beyond a certain length, denoted here as SL_y, the passive force-length relationship levels off appreciably and may even drop slightly, probably due to disruption of the thin-thick filament anchorage (Linke et al. 1996; Wang et al.

1991). Here, we found that the SL_y was ~3.9 and ~4.6 μm in rat and human type II fibres, respectively, and also that passive force was irreversibly depressed after overstretching beyond these SL_y (Fig. 6). These values may be important in regard to the impact of eccentric exercise on the muscle. Talbot and Morgan (1996) found that some sarcomeres are 'popped' following eccentric contractions, possibly because of disruption of the thin-thick filament anchorage by overstretch (Talbot and Morgan 1996). The SL_y values found here may aid understanding of the exact conditions required for such sarcomere popping, and possibly also help guide eccentric training in humans, although further studies are clearly required.

Physiological relevance

Previous studies have shown that *S*-glutathionylation of TnI occurs with *in vivo* muscle activity (Mollica et al. 2012; Watanabe et al. 2015). Here, we provide evidence that *S*-glutathionylation, presumably of titin, does decrease passive force in skeletal muscle. Interestingly, a recent study has indicated that titin contributes not only to passive force, but also to active force production, by assisting the sliding of the thick filament (Rivas-Pardo et al. 2016), implying that *S*-glutathionylation of titin may cause a decrease in active force production as well as passive force production. Titin has to be stretched to expose the cryptic cysteine residues for *S*-glutathionylation to occur (Alegre-Cebollada et al. 2014). Sufficient stretching of fibres may possibly occur during normal muscular activity or might primarily occur with eccentric contractions, when some sarcomeres could be stretched to near or beyond SL_y (Talbot and Morgan 1996). Future studies examining the impact of *S*-glutathionylation of titin with eccentric contractions could provide valuable physiological insight, with potential implication for strategies for efficient eccentric training.

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Table

Table 1. Parameters of exponential fit of passive force-length relationship in rat and human fibres. Values are means ± SE. The start point of the exponential fit (SL_e) defined as SL where fibre just started to produce detectable passive force. The *A* and λ were the scaling constant and the length constant of exponential fit, respectively (see text). Shapiro-Wilk normality test showed all data sets normally distributed, and statistical differences examined with Student's unpaired t-test. No significant differences between type I and type II fibres, in either rat or human. † Value in human fibres significantly different from that in same fibre type in rat ($P<0.05$; two-tailed t-test).

Figures

Fig. 1. Representative western blotting of myosin heavy chain (MHC) I, IIa and IIx. Following physiological experiments, each individual skinned fibre segment from human muscle was collected and analyzed by western blotting. All six fibres from one subject (male, 29 years old, 181 cm height, 66 kg body weight) shown. Upper three panels show blots obtained by probing successively for MHCIIx, MHCIIa and MHCI. Bottom panel shows myosin band region on Stain Free gel, and labels beneath indicate assigned fibre type.

Fig. 2. Representative example of passive force responses with each treatment in rat type II fibre. *A,* passive force responses after various indicated treatments; numbers above traces indicate sarcomere length in μm. Each treatment applied in relaxing solution, and all treatments except for '20 mM GSSG without stretching' were performed whilst stretching the fibre to $SL_{20\%\ max}$ (see text) (to 3.61 μm in this fibre). *B,* passive force-length relationships in *A.* Each set of data points was fitted with a simple exponential function (see Materials and Methods) (R^2>0.97). The starting point (SL_e) of each curve, defined as the SL where the fibres just start to produce detectable passive force, was 2.49 μm. The length constant parameter, λ, for fitted each curve was 0.26, 0.28, 0.28, 0.27 and 0.21 μm, before any treatment, after initial DTT, after GSSG with no stretch, after GSSG with stretch, and after second DTT, respectively. *A* value of fit was 0.26, 0.28, 0.28, 0.27 and 0.21 before treatment, after initial DTT, after GSSG with no stretch, after GSSG with stretch, and after second DTT, respectively.

Fig. 3. Effect of exposure to dithiothreitol and oxidized glutathione on passive force production. Passive force measured upon stretching fibre to SL that elicited the equivalent of ~20% of maximum Ca^{2+}-activated force in the initial control conditions ($SL_{20\%\ max}$). Each freshly skinned fibre was exposed to 10 mM DTT for 10 min, and subsequently treated with

20 mM GSSG for 15 min, as in Fig. 2. In some rat type II fibres, the effect of applying GSSG with the fibre unstretched, was also examined. '*n*' denotes the number of fibres and '*N*' denotes the number of subjects from which the fibres were obtained. Values are means ± SE. Shapiro-Wilk normality test performed to determine whether distribution of data set was normal; all data sets showed normal distribution. Paired two-tailed t-test used to examine statistical significance. $SL_{20\%\ max}$ was 3.72 ± 0.02 μm, 3.72 ± 0.05 μm, 4.21 ± 0.07 μm and 4.23 ± 0.04 μm in rat type II, rat type I, human type II and human type I, respectively. $^{a}P<0.05$ vs. control (two-tailed paired t-test). Rat type I and II fibres obtained from soleus and EDL muscles respectively, with fibre type verified by response to Sr^{2+} (see Materials and Methods). Human fibres from *vastus lateralis* muscle, with the type determined by western blotting.

Fig. 4. Passive force-length relationship in type I (red) and type II (blue) fibres of rat (A) and human (B). Data points were fitted with an exponential function ($R^2>0.98$). Mean values of fit parameters shown in Table 1. Numbers of fibres: 8, 8, 13 and 12 in rat type I, rat type II, human type I and human type II, respectively. Different symbols used to denote data points in different individual fibres.

Fig. 5. Subject-dependent effect in human type II fibres. *A,* passive force-length relationship in fibres from different subjects (Sub.). Data points fitted with exponential function. Three fibres (green circle, green star and green triangle) were type IIa/x and other 10 fibres were pure type IIa. *B,* correlation between GSSG-induced force reduction and the length constant (λ) of fit to passive force-length relationship in that fibre before treatment.

Fig. 6. Effect of overstretch on passive force in rat and human type II fibre. *A,* typical passive force responses at each sarcomere length (SL) in rat type II fibre. Fibre stretched for

665 2 min at SL shown above trace (in μm). Passive force at 3.4 μm SL (initial level marked with
666 dashed line) was decreased after stretching to long SLs (subsequent passive force values
667 indicated by white arrows). *B*, passive force-length relationship in *A*. A given color represents
668 passive force measured at a particular long SL and at 3.2 μm and 3.4 μm SL after the stretch
669 to that long SL. Passive force-length relationship increased exponentially until ~4.0 μm SL,
670 and then the curve started to level off at longer SLs, i.e., 4.5 μm and 5.1 μm. SL_y defined as
671 the SL where exponential curve (dash red line) intersects a line joining the higher SL data
672 (black line). *C*, values of SL_y in human and rat type II fibres. Four fibres examined from two
673 rats and four fibres from two human subjects. $^{a}P < 0.05$ vs. Rat (two-tailed t-test).

Figure

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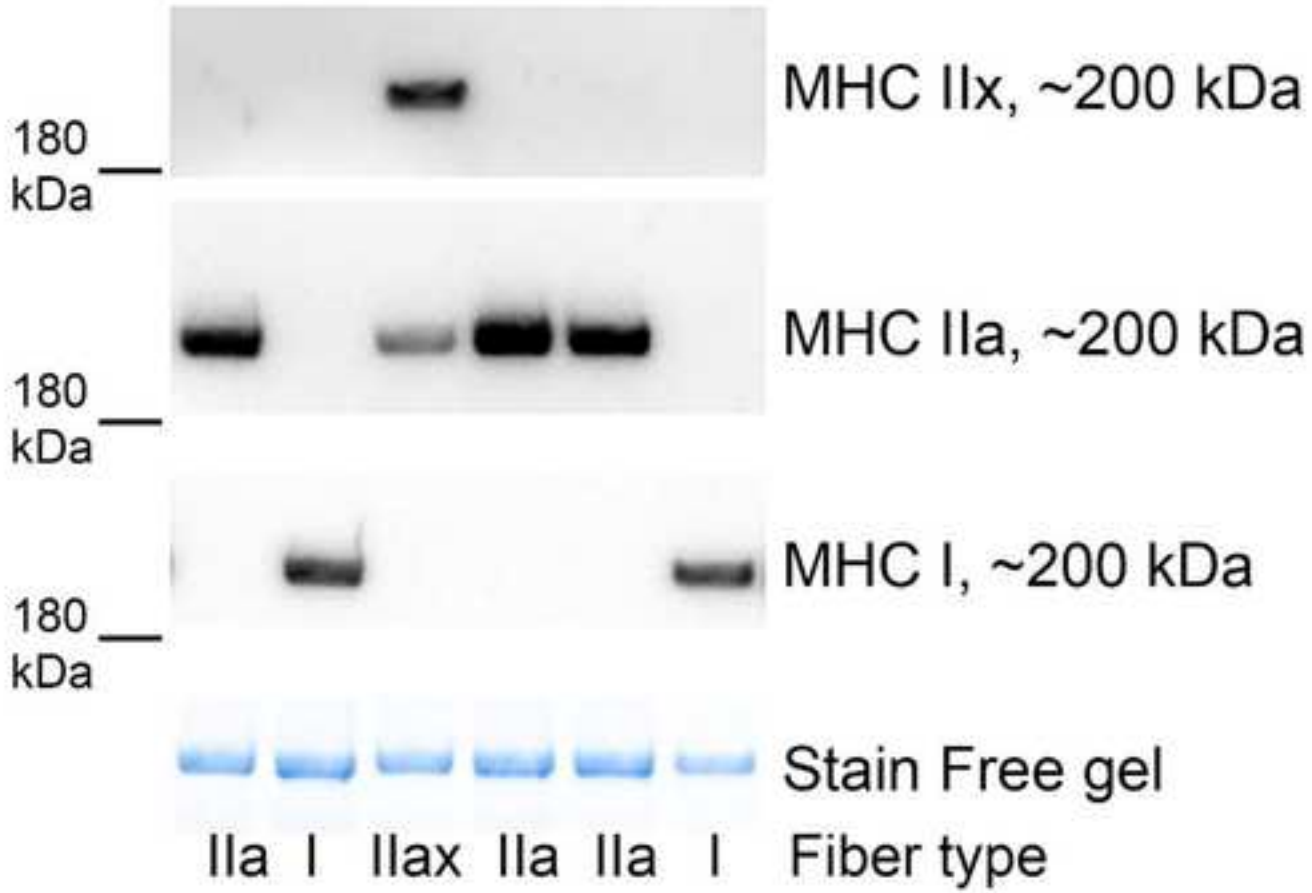

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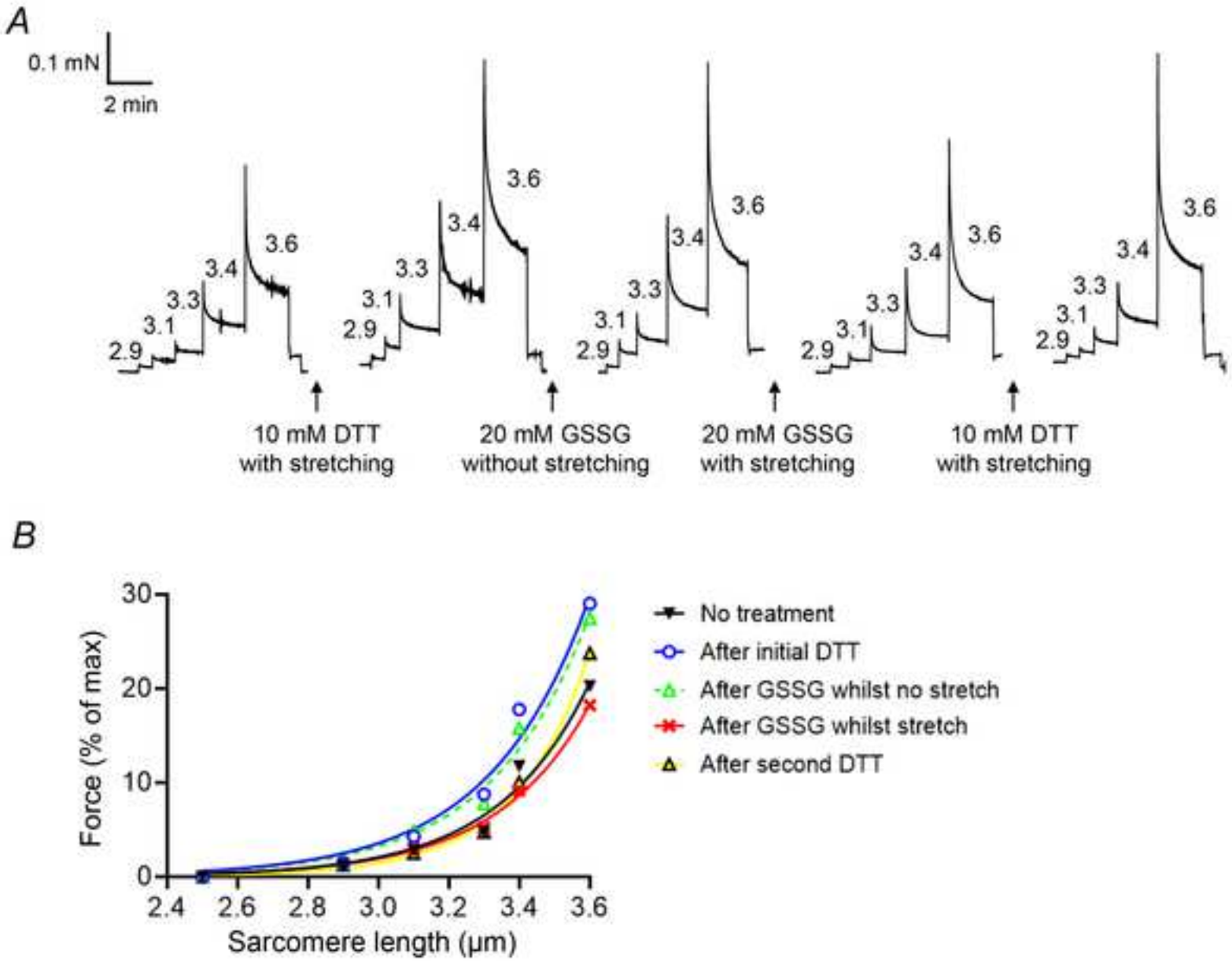

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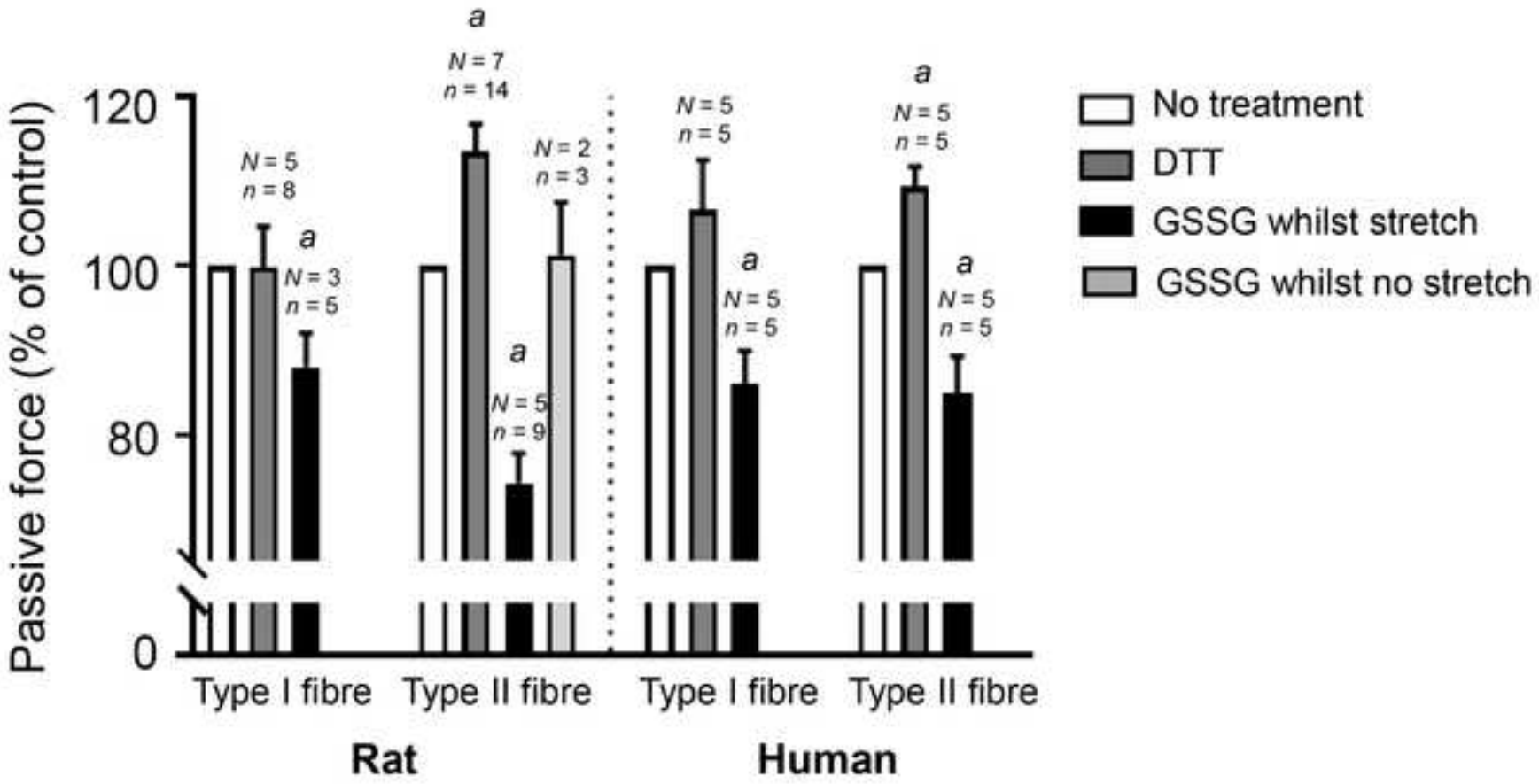

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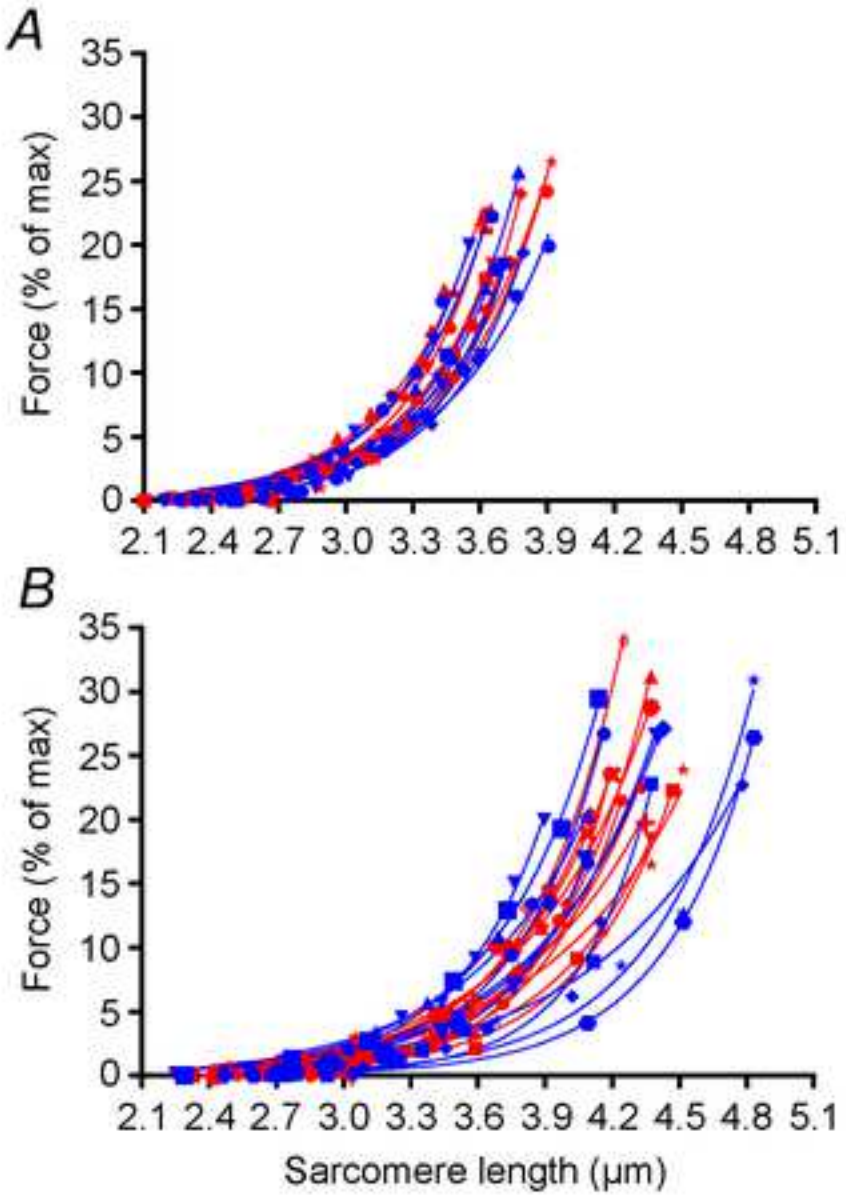

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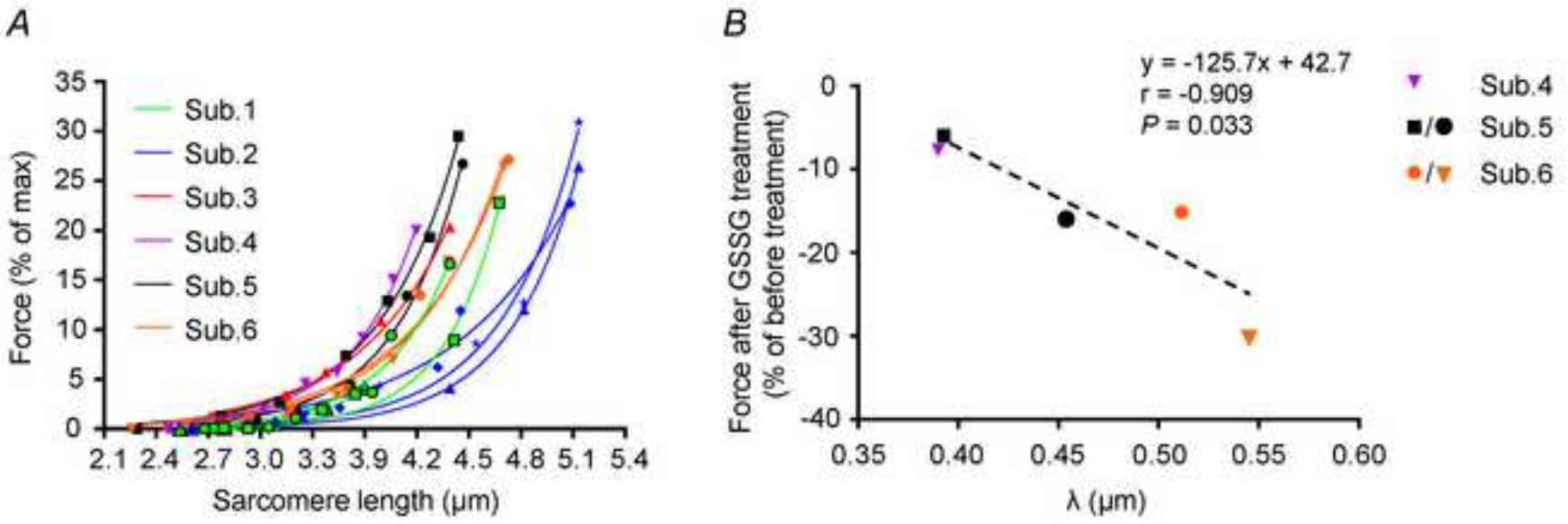

Fig. 5

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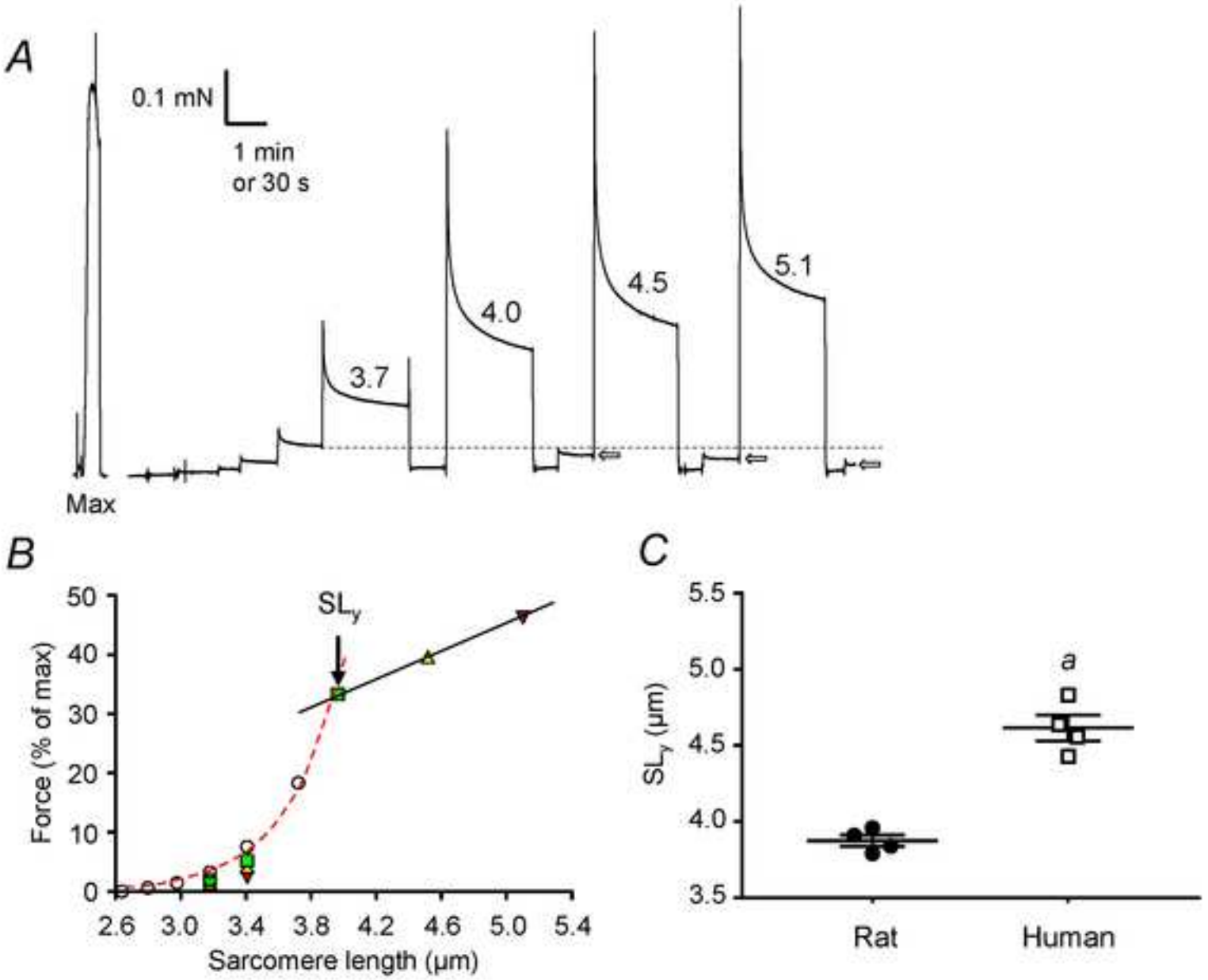

Fig. 6

Table 1. Parameters of exponential fit of passive force-length relationship in rat and human fibres.

Parameters	Rat type II (n = 8, N = 5)	Rat type I (n = 8, N = 5)	Human type IIa + IIa/x (n = 13, N = 6)	Human type I (n = 12, N =7)
SL_e (μm)	2.36 ± 0.05	2.41 ± 0.07	2.53 ± 0.04†	2.63 ± 0.07†
A	0.59 ± 0.08	0.85 ± 0.20	0.59 ± 0.12	0.79 ± 0.12
λ (μm)	0.38 ± 0.01	0.38 ± 0.01	0.46 ± 0.04†	0.47 ± 0.02†

Values are means ± SE. The start point of the exponential fit (SL_e) was defined as SL where fibre just started to produce detectable passive force. A and λ are the scaling constant and the length constant of exponential fit, respectively (see text). Shapiro-Wilk normality test showed all data sets were normally distributed, and statistical differences were examined with Student's unpaired t-test. No significant differences found between type I and type II fibres, in either rat or human. † Value in human fibres significantly different from that in same fibre type in rat (P<0.05; two-tailed t-test).