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Contractile properties and sarcoplasmic reticulum calcium content in type I and type II skeletal muscle fibres in active aged humans

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Key points

- Muscle weakness in old age is due in large part to an overall loss of skeletal muscle tissue, but it remains uncertain how much also stems from alterations in the properties of the individual muscle fibres.
- This study examined the contractile properties and amount of stored intracellular calcium in single muscle fibres of Old (70 ± 4 years) and Young (22 ± 3 years) adults.
- The maximum level of force production (per unit cross-sectional area) in fast twitch fibres in Old subjects was lower than in Young subjects, and the fibres were also less sensitive to activation by calcium.
- The amount of calcium stored inside muscle fibres and available to trigger contraction was also lower in both fast- and slow-twitch muscle fibres in the Old subjects.
- These findings indicate that muscle weakness in old age stems in part from an impaired capacity for force production in the individual muscle fibres.

Abstract This study examined the contractile properties and sarcoplasmic reticulum (SR) Ca^{2+} content in mechanically skinned vastus lateralis muscle fibres of Old (70 ± 4 years) and Young (22 ± 3 years) humans to investigate whether changes in muscle fibre properties contribute to muscle weakness in old age. In type II fibres of Old subjects, specific force was reduced by ∼17% and Ca^{2+} sensitivity was also reduced (pCa_{50} decreased ∼0.05 pCa units) relative to that in Young. *S*-Glutathionylation of fast troponin I (TnI_f) markedly increased Ca^{2+} sensitivity in type II fibres, but the increase was significantly smaller in Old *versus* Young (+0.136 and +0.164 pCa unit increases, respectively). Endogenous and maximal SR Ca^{2+} content were significantly smaller in both type I and type II fibres in Old subjects. In fibres of Young, the SR could be nearly fully depleted of Ca^{2+} by a combined caffeine and low Mg^{2+} stimulus, whereas in fibres of Old the amount of non-releasable Ca^{2+} was significantly increased (by > 12% of endogenous Ca^{2+} content). Western blotting showed an increased proportion of type I fibres in Old subjects, and increased amounts of calsequestrin-2 and calsequestrin-like protein. The findings suggest that muscle weakness in old age is probably attributable in part to (i) an increased proportion of type I fibres, (ii) a reduction in both maximum specific force and Ca^{2+} sensitivity in type II fibres, and also a decreased ability of *S*-glutathionylation of TnI_f to counter the fatiguing effects of metabolites on Ca^{2+} sensitivity, and (iii) a reduction in the amount of releasable SR Ca^{2+} in both fibre types.

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Abbreviations CaBAPTA, calcium bound to BAPTA; CaEGTA, calcium bound to EGTA; CSQ, calsequestrin; *h*, Hill coefficient; HDTA, hexa-methylene-diamine-tetraacetate; MHC, myosin heavy chain; *n*, number of fibres; *N*, number of subjects; pCa, $-\log_{10}[Ca^{2+}]$; pCa_{50}, pCa producing half-maximal force; pSr, $-\log_{10}[Sr^{2+}]$; SR, sarcoplasmic reticulum; TnC, troponin C; TnI_f, fast troponin I.

Introduction

Skeletal muscle strength declines in old age in humans even when maintaining an active lifestyle (Miller *et al.* 2014). Much of this decline is due to an overall loss of muscle tissue and to a decrease in the proportion of type II fibres (Poggi *et al.* 1987; Marzani *et al.* 2005; Staunton *et al.* 2012), but many studies, though not all (e.g. Trappe *et al.* 2003), have concluded that the decline in strength is also due to impaired performance at the single fibre level, involving decreases in specific force, velocity of shortening or Ca^{2+} sensitivity of the contractile apparatus (Frontera *et al.* 2000; D'Antona *et al.* 2003; Yu *et al.* 2007; Hvid *et al.* 2013*a*) or decreased release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Delbono *et al.* 1995).

The molecular basis of contractile apparatus dysfunction occurring with age is uncertain. One possibility is that it is due to oxidant-induced changes to the contractile apparatus. Reactive oxygen and nitrogen species are generated in skeletal muscle during normal contraction as well as in pathological conditions (Powers & Jackson, 2008; Reid, 2008; Reid & Moylan, 2011), and there is a substantial amount of evidence of increased oxidative damage in muscle with age (Fano *et al.* 2001; Fulle *et al.* 2004; Prochniewicz *et al.* 2007; Jackson & McArdle, 2011; Tohma *et al.* 2014). Exposure of isolated muscles or intact or skinned fibres to superoxide, peroxynitrite or high and prolonged concentrations of H_2O_2 causes a reduction in specific force and/or Ca^{2+} sensitivity (Andrade *et al.* 1998; Supinski *et al.* 1999; Plant *et al.* 2000; Callahan *et al.* 2001*b*; Darnley *et al.* 2001; Lamb & Posterino, 2003; Prochniewicz *et al.* 2008; Dutka *et al.* 2011*a*). Mild oxidative treatment of intact fibres on the other hand causes an increase in Ca^{2+} sensitivity (Andrade *et al.* 1998), resulting from reversible *S*-glutathionylation of fast troponin I (TnI_f) (Dutka *et al.* 2011*b*; Lamb & Westerblad, 2011; Mollica *et al.* 2012), which is likely to be an important mechanism helping delay the onset of muscle fatigue during exercise (Mollica *et al.* 2012). Strong oxidative treatment, however, irreversibly blocks this Ca^{2+}-sensitizing effect of *S*-glutathionylation (Murphy *et al.* 2008), probably by causing irreversible oxidation of the critical cysteine on TnI_f (Lamb & Westerblad, 2011).

Decreased SR Ca^{2+} release in muscle fibres with ageing could arise from dysfunction of the voltage sensors/dihydropyridine receptors (DHPRs) in the transverse tubular system, or dysfunction of the ryanodine receptors (RyRs) in the SR (Andersson *et al.* 2011), or disrupted communication between the DHPRs and the RyRs (Delbono *et al.* 1995). Alternatively, decreased SR Ca^{2+} release could result simply from there being less Ca^{2+} stored in the SR in the fibres of aged individuals. Recently we used a fibre-lysing technique (Fryer & Stephenson, 1996) to measure the SR Ca^{2+} content of muscle fibres of young adults (Lamboley *et al.* 2013). There are no data to date, however, to show whether the SR Ca^{2+} content of muscle differs between old and young individuals.

Here, we use skinned muscle fibres obtained from fresh biopsies of the vastus lateralis muscle to compare the contractile apparatus properties and SR Ca^{2+} content in Old (70 ± 4 years) and Young (22 ± 3 years) adults. The aims of the experiments were to determine whether there were any differences between muscle of Old and Young adults with respect to: (i) the specific force and Ca^{2+} sensitivity in type I and type II fibres, and the effects of reversible oxidative modification, (ii) the endogenous and maximal Ca^{2+} content of type I and type II fibres and the amount remaining after depleting the SR with a potent caffeine–low Mg^{2+} stimulus, and (iii) the relative content of the primary SR Ca^{2+}-buffering molecules calsequestrin 1 (CSQ1) and calsequestrin 2 (CSQ2).

Methods

Participants

This study was approved by the Human Research Ethics Committees of Victoria University and La Trobe University, and conforms to the *Declaration of Helsinki*. After reading all information, a total of sixteen 'Young' (10 males and 6 females) and twenty 'Old' adult participants (13 males and 7 females) gave signed informed consent. The physical characteristics of the Old group (mean ± SD) were: age 70 ± 4 years, height 170 ± 9 cm and body mass 74 ± 12 kg. Those of the Young group were: age 22 ± 3 years, height 176 ± 12 cm, body mass 75 ± 12 kg. Apart from age ($P < 0.05$), these physical characteristics did not significantly differ between groups ($P > 0.05$). All participants were healthy and recreationally active but were not specifically trained in any sport. Prior to inclusion in the study, the current level of self-reported physical activity of participants

was determined with a physical activity questionnaire about the preceding 7 days, and subjects in the Young and Old groups were recruited with a similar number of self-reported hours of physical activity. The type of physical activity reported by the participants ranged from walking and gardening to running, cycling and light weight lifting. The questionnaire included questions about all exercise, including occupational tasks, household duties and sports activities and the exercise was split into three categories: moderate, hard and very hard exercise. Overall, there was no significant difference between Young and Old participants either in the number of hours of physical activity conducted in total (6.8 ± 1.7 and 8.2 ± 4.4 h, respectively) or in the three different exercise categories. One limitation of this questionnaire is that it is self-reported and Old and Young adults may have different perceptions as to what is hard and very hard (despite information and examples provided on the questionnaire). All Old participants were physically fit and were cleared by their medical doctor to participate in high-intensity exercise (up to 95% peak heart rate). Participants were excluded from the study if they had any of the following conditions: diabetes (Type 1 or 2), chronic heart disease, severe hypertension, or were severely overweight/obese (body mass index > 30), or had any recent significant injury or other contra-indications that would impede their ability to safely perform exercise.

Muscle biopsy

Experiments were performed with muscle tissue obtained from a thigh muscle biopsy in rested conditions. After injection of a local anaesthetic (1% Xylocaine) 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, with one part placed in paraffin oil (Ajax Chemicals, Sydney, Australia) for fibre dissection (see below) and the remaining part stored in liquid nitrogen for later analysis.

Skinned fibre preparation and force recording

The muscle biopsy was pinned at resting length in a Petri dish containing paraffin oil and kept cool (~10°C) on an icepack. Individual fibre segments were mechanically skinned as described previously (Murphy *et al.* 2009*b*; Lamboley *et al.* 2013) and pinned out unstretched under oil, with the diameter being measured at three places along the fibre. Fibre cross-sectional area was calculated assuming an ellipsoidal profile with dimensions corresponding to the largest and smallest diameter measurements. The skinned fibre segment was then mounted at 120% of resting length on a force transducer (AME801, SensoNor, Horten, Norway) and placed in a Perspex bath containing 2 ml of the standard K^+-based solution broadly mimicking the intracellular milieu (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 specified otherwise. The standard K-HDTA solution contained (in mM): hexa-methylene-diamine-tetraacetate ($HDTA^{2-}$), 50 (Fluka, Buchs, Switzerland); total ATP, 8; Na^+, 36; K^+, 126; total Mg^{2+}, 8.5 (giving 1 mM free $[Mg^{2+}]$); creatine phosphate, 10; total EGTA, 0.05; Hepes, 90; pH 7.1 and pCa ($-\log_{10}[Ca^{2+}]$) ~7.1, except where stated. Where required, the SR of the skinned fibre was totally depleted of all releasable Ca^{2+} by exposure to the 'full release solution', which was similar to the K-HDTA solution but with 30 mM caffeine, 0.05 mM free Mg^{2+} (total Mg^{2+} of 2.1 mM) and 0.5 mM free EGTA (pCa 8.5) present to chelate released Ca^{2+}. Where required, the SR was maximally loaded with Ca^{2+} by exposing the fibre for 4 min to a load solution, which was the same as the standard K-HDTA solution but with the pCa buffered at 6.7 with 1 mM total EGTA (0.5 mM EGTA with Ca bound (CaEGTA) and 0.5 mM free EGTA) (see Lamboley *et al.* 2013).

The properties of the contractile apparatus were examined using heavily Ca^{2+}-buffered solutions in which all HDTA was replaced by EGTA (relaxing solution) or CaEGTA (maximum Ca^{2+}-activating solution), as described previously (Lamb & Posterino, 2003; Lamboley *et al.* 2013). The relaxing solution contained 50 mM EGTA and no added Ca^{2+} (pCa > 9) and the maximum Ca^{2+}-activating solution contained 49.5 mM Ca^{2+} (pCa 4.7), with total Mg^{2+} of 10.3 and 8.1 mM, respectively, to maintain the free $[Mg^{2+}]$ at 1 mM. These two solutions were mixed in appropriate ratios to produce solutions with pCa in the range 6.7–4.7. In addition, a strontium-based solution (at pSr ($-\log_{10}[Sr^{2+}]$) 5.2) was made by mixing relaxing solution (50 mM EGTA) with Sr-EGTA solution similar to the maximum Ca^{2+}-activating solution.

The BAPTA solution used to pre-equilibrate fibres before lysing (see below) was similar to the standard K-HDTA solution but had no EGTA and instead had 0.025–1.8 mM BAPTA, added from a 47 mM BAPTA stock solution. The BAPTA stock solution was titrated with Ca^{2+} using a Ca^{2+}-sensitive electrode (Orion Research, Boston, MA, USA) to establish the exact amount of BAPTA

present, and was the same stock as used in our matching study in Young subjects (Lamboley *et al.* 2013).

Contractile apparatus experiments

The force–Ca^{2+} relationship was determined by exposing the skinned fibre segment to a sequence of heavily buffered solutions at progressively higher free [Ca^{2+}] (50 mM CaEGTA–EGTA, pCa > 9–4.7), as shown in Fig. 1, with maximum force defined as that elicited at pCa 4.7. Isometric force produced at each [Ca^{2+}] was expressed as a percentage of the corresponding maximum force and analysed by fitting a Hill curve using GraphPad Prism 6 software to ascertain the pCa_{50} (pCa at half-maximum force) and Hill coefficient (h) for each sequence. In the experiments on the effects of DTDP–GSH treatment (i.e. 100 μM DTDP for 5 min followed by 5 mM GSH for 2 min) on Ca^{2+} sensitivity, the change in pCa_{50} with DTDP–GSH treatment was defined in each fibre as the average of that observed with the treatment itself and upon subsequent reversal by DTT treatment, so as to take into account the small progressive decrease in Ca^{2+} sensitivity occurring when subjecting a fibre to repeated force–pCa staircases (see Fig. 1).

Experimental protocol for quantifying total Ca^{2+} content in the fibre

As described previously (Fryer & Stephenson, 1996; Owen *et al.* 1997; Lamboley *et al.* 2013), the total amount of Ca^{2+} present in a fibre can be quantified by briefly pre-equilibrating the skinned fibre in a solution with a known concentration of the fast calcium-buffer BAPTA and then transferring the fibre to an emulsion of 10% Triton X-100 and paraffin oil (TX–oil) to lyse all membranous compartments and release any Ca^{2+} from within the fibre. The skinned fibre was first placed in the standard solution K-HDTA for 2 min to wash out all the diffusible Ca^{2+}-binding proteins present endogenously in the cytoplasm. This solution had very little contaminating Ca^{2+} or EGTA, so that there was negligible additional Ca^{2+} loading, but any Ca^{2+} leaking out of the SR was readily resequestered by the SR and retained within the fibre. The skinned fibre was then equilibrated for 20 s in a solution with a set [BAPTA] before being placed in a freshly triturated emulsion of Triton X-100 in paraffin oil (TX–oil) (10% v/v). The Ca^{2+} liberated by the membrane lysing rapidly binds to the known amount of BAPTA present within the fibre and to other sites, predominantly troponin C (TnC). If the pre-equilibrating [BAPTA] was chosen such that the fibre produced a finite, non-maximal force response upon lysis, then the total amount of Ca^{2+} present in the fibre could be calculated in absolute terms from the [BAPTA] in the equilibration solution and the magnitude of the force response (see below and Lamboley *et al.* 2013). Other skinned fibre segments, prior to the TX–oil lysing, were (i) fully depleted of their endogenous SR Ca^{2+} content by a 1 min exposure to the full release solution, or (ii) loaded to their maximal SR Ca^{2+} capacity

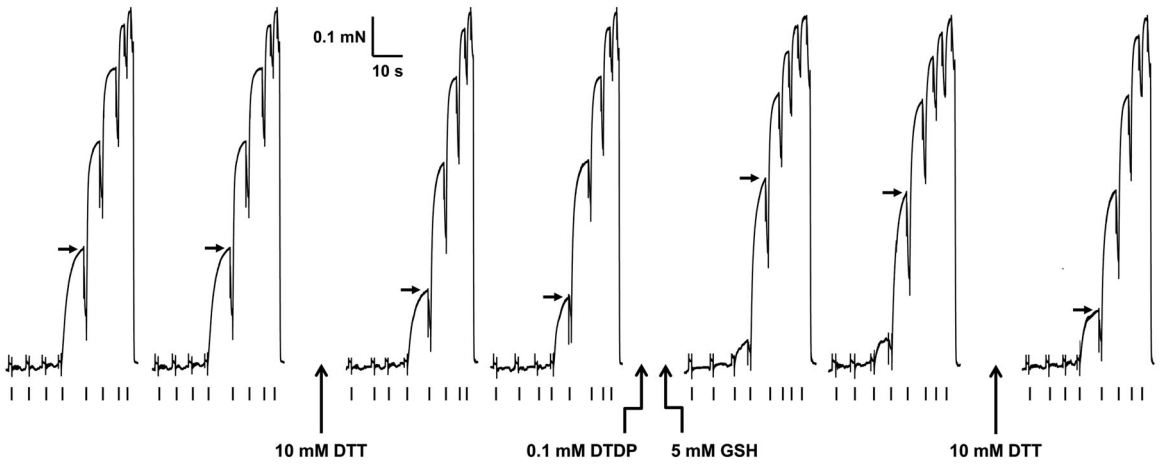

Figure 1. Effect of DTT and DTDP–GSH exposures on Ca^{2+} sensitivity of contractile apparatus in type II vastus lateralis fibre of an Old subject
Force responses elicited by directly activating the contractile apparatus with heavily Ca^{2+}-buffered solutions with progressively higher free [Ca^{2+}] (pCa of successive solutions: > 9, 6.7, 6.4, 6.22, 6.02, 5.88, 5.75, 5.48, 4.7, then back to > 9, marked by ticks under each force trace). Force–pCa staircases were elicited twice successively for each of the four conditions: (1) control (untreated), (2) following treatment with DTT (10 mM, 5 min), (3) following *S*-glutathionylation treatment by applying DTDP (0.1 mM, 5 min) and then GSH (5 mM, 2 min), and finally (4) after a further treatment with DTT (only one force–pCa staircase shown). All treatments applied in relaxing solution (pCa > 9) and fibre washed for > 1 min in relaxing solution before each force staircase. Horizontal arrows indicate force level produced at pCa 6.02 on each staircase. For conditions 1–4, the average pCa_{50} values were 5.922, 5.905, 6.010 and 5.880, respectively.

by a 4 min exposure to standard load solution (pCa 6.7, buffered with 1 mM total EGTA) (see 'Skinned fibre solutions').

Calculation of Ca^{2+} release from lysing experiment

The total Ca^{2+} content within the fibre at the time of lysis ($[Ca^{2+}]_T$), expressed in millimoles per litre total fibre volume (in keeping with previous studies: Fryer & Stephenson, 1996; Owen *et al.* 1997), could be calculated as the sum of (i) the Ca^{2+} bound to BAPTA, (ii) the Ca^{2+} bound to all other high affinity binding sites in the fibre (predominantly TnC), and (iii) the free Ca^{2+} in the myoplasm ($[Ca^{2+}]$), as described in detail previously (Lamboley *et al.* 2013). In brief, the total amount of Ca^{2+} within a given fibre ($[Ca^{2+}]_T$) was calculated as follows:

(1) The cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]$, in molar units) within the fibre at the peak of the force response elicited upon lysis was calculated from the relationship between force and $[Ca^{2+}]$ for that fibre type, which was defined as being the Hill curve with pCa_{50} and Hill coefficient equal to the mean of the values found from the individual fits of the data for each fibre of the specified type and age group (i.e. mean values in Table 1; Fig. 3 plots curves for mean values found for type II fibres). The $[Ca^{2+}]$ was calculated analogously to equations (1) and (2) in Lamboley *et al.* (2013).
(2) The effective [BAPTA] within the fibre was taken as being 1.13 times the [BAPTA] of the pre-equilibration solution, to account for the swelling of the fibre when initially placed in solution, and also the fibre volume to which BAPTA was not accessible (i.e. that occupied by the SR, t-tubular system and mitochondria etc.) (Fryer & Stephenson, 1996).
(3) The percentage of BAPTA with bound Ca^{2+} (%CaBAPTA) was determined from the size of the force response, the relevant force–$[Ca^{2+}]$ relationship (Table 1) and the known Ca^{2+}-binding properties of BAPTA (Harrison & Bers, 1987) (see Owen *et al.* 1997; Posterino & Lamb, 2003), calculated analogously to that set out in equations (3) and (4) in Lamboley *et al.* (2013).
(4) The total possible increase in Ca^{2+} binding to TnC was taken as 70 and 140 μmol l^{-1} binding to the Ca^{2+}-specific sites in type I and type II fibres, respectively, and a further 140 μmol l^{-1} binding to the non-Ca^{2+}-specific sites present in both (i.e. 1 or 2 Ca^{2+}-specific sites and 2 non-specific sites per TnC molecule) (Fryer & Stephenson, 1996). For experiments where a fibre was pre-equilibrated in [BAPTA] > 0.5 mM (and hence pCa > 8.3), the amount of Ca^{2+} binding to TnC (CaTnC) (in mmol l^{-1}) as a function of force was calculated based on the data of Robertson *et al.* (1981) and Fuchs (1985), as described previously (Lamboley *et al.* 2013). In cases where the [BAPTA] in the pre-equilibration solution was very low (≤0.05 mM), the pCa of the solution was ~7 or lower, and so the non-specific sites on TnC were assumed to have bound Ca^{2+} already in the pre-equilibration solution before the lysing.
(5) Ca^{2+} binds to the ATP and HDTA present, and the total of these and the free $[Ca^{2+}]$ was estimated as being ~9.6 × $[Ca^{2+}]$ (Posterino & Lamb, 2003).
(6) Finally, 0.015 mmol l^{-1} was deducted from the total to take into account the contaminating Ca^{2+} present in the BAPTA pre-equilibration solution.

The total Ca^{2+} content within the fibre at the time of the lysis (expressed in millimoles per litre fibre volume) was thus calculated as being:

$$[Ca^{2+}]_T = 1.13 \times [\text{BAPTA}] \times \%\text{CaBAPTA}/100 + \text{CaTnC} + 9.6 \times [Ca^{2+}]/1000 - 0.015$$

Muscle homogenates

Homogenates. Frozen muscle samples were used for whole muscle homogenates, similar to previously described (Murphy *et al.* 2011). At a ratio of 1:20 (w/v), samples (3–15 mg) were placed in ice-cold Na^+ homogenizing solution, which was similar to the relaxing solution described in 'Skinned fibre solutions', except the K^+ was replaced with Na^+, and protease inhibitor cocktail (Roche Diagnostics, Sydney, NSW, Australia) was added. Samples were homogenized (~3 × 8 s bursts with Polytron homogenizer), then vortexed. To obtain a 'whole muscle homogenate', a 20 μl aliquot was diluted in 3× SDS loading buffer (2:1 v/v) which contained 0.125 M Tris-HCl, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol and ~0.001% bromophenol blue (pH 6.8) to a final concentration of 2.5 μg wet weight μl^{-1} and stored at −80°C until Western blotting.

Cytoskeletal proteins. To obtain the cytoskeletal pool of proteins in a given sample, a 5 μl aliquot from each original homogenate was diluted (1:20, v/v) in Na^+ homogenizing solution with 1% Triton X-100 added and the sample spun (14,000 *g*, 5 min, 4°C). The pellet (cytoskeletal fraction) was suspended in 3× solubilizing buffer which had been diluted 1:2 in Na^+ homogenizing solution (1× solubilizing buffer). All these samples from Young and Old individuals were separated on a 4–15% Criterion Stain-Free gel (BioRad, Sydney, NSW, Australia) and visualized following UV activation. The myosin (~210 kDa) and actin (~43 kDa) bands were expressed relative to the total protein in a given lane.

Single fibres. Following physiological experiments, individual skinned fibre segments were placed in 10 μl

1× solubilizing buffer and stored at −80 °C until analysed by Western blotting.

Western blotting and fibre typing

The entire set of Young and Old muscle homogenates were run on the same gel, along with a 3–5 point calibration curve which consisted of different amounts of a mixed standard homogenate made with the muscle homogenates from a number of individuals. A selection of single fibres from both Young and Old individuals were always run on a gel together, along with a calibration curve. Similar to that described previously (Murphy, 2011; Lamboley *et al.* 2013), total protein was separated on either 10% (whole muscle) or 4–15% (single fibres) Criterion Stain-Free precast gels (Bio-Rad; 1 h at 200 V) and an image taken of the UV-activated gel prior to transfer to nitrocellulose membrane (30 min at 100 V). Following treatment with Antibody Extender Solution (ThermoFisher Scientific, Scoresby, Vic., Australia), and blocking (5% skimmed milk in Tris-buffered saline with Tween, TBST, 1–2 h), membranes were probed separately with primary antibodies, all diluted in 1% BSA in phosphate-buffered saline with Tween, being constantly rocked overnight at 4 °C and for 2 h at room temperature. Antibodies were: calsequestrin 1 and calsequestrin-like proteins (mouse monoclonal, Abcam, cat. no. ab2824, 1 in 2000), calsequestrin 2 (rabbit, Abcam, cat. no. ab3516, 1 in 1000), myosin heavy chain II (MHCIIa, mouse IgG, Developmental Studies Hybridoma Bank (DSHB), cat. no. A4.74, 1 in 400), MHCI (mouse IgM, DSHB, cat. no. A4.840, 1 in 100) and MHCIIx (mouse IgM, DSHB, cat. no. 6H1, 1 in 100). The horseradish peroxidase secondary antibodies used were goat anti mouse (1 in 20,000; Thermo Fisher Scientific, cat. no. PIE31430), goat anti rabbit (1 in 60,000; Thermo Fisher Scientific, cat. no. PIE31460) and goat anti-mouse IgM (1 in 20,000; 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). Proteins of interest were expressed relative to the total protein seen on the Stain-Free gel. The MHCIIa antibody used here appeared to specifically label MHCIIa and not MHCIIx (B. Frankish & R. M. Murphy, unpublished observations). Each single fibre was Western blotted for both MHCIIa and MHCI; all fibres showed labelling to one or the other antibody, with only a small proportion showing labelling to both (defined as 'mixed' fibres, see Results). Single fibres were not routinely blotted for MHCIIx, though it could be inferred from the above that there were no pure IIx fibres. Consequently, fibres were defined as being type I or type II (which included both IIa and IIa/x fibres) or mixed.

The responsiveness of fibres to a strontium-based solution at pSr 5.2 was also examined following completion of the relevant force measurements; as in our previous studies on human fibres (Lamboley *et al.* 2013, 2014), it was found that the responsiveness to Sr^{2+} was always in very close accord with the fibre type subsequently identified by Western blotting with the pSr 5.2 solution eliciting > 70% of maximum Ca^{2+}-activated force in type I fibres, < 6% of maximum force in type II fibres, and an intermediate level in mixed fibres.

Statistics

Values are presented individually or as mean ± SD, with n denoting the number of fibres examined and N the number of subjects. Statistical significance ($P < 0.05$) was determined with Student's t test or the non-parametric Mann–Whitney rank test where data were not normally distributed, or two-way ANOVA where indicated.

Results

We measured force responses in a total of 105 skinned muscle fibres from 16 Young adults and 231 muscle fibres from 20 Old adults. Subsequent Western blotting of myosin heavy chain (MHC) showed that the sample of Young fibres consisted of 54 type I, 48 type II and 3 'mixed' fibres (fibres containing both MHCI and MHCII, see Methods), and the sample of Old fibres consisted of 146 type I, 71 type II, and 14 mixed fibres. Results for the mixed fibre group are not presented here because the proportion of MHCI and MHCII varied greatly between different fibres; we note nevertheless that the properties of a mixed fibre where one MHC isoform predominated in general resembled the properties of fibres containing solely that MHC isoform.

Contractile properties of fibres and the effects of DTT and *S*-glutathionylation

The Ca^{2+} sensitivity and maximum force production in each skinned fibre was assessed by activating the contractile apparatus in a series of solutions with the free [Ca^{2+}] heavily buffered at progressively higher levels, from < 1 nM up to 20 μM (i.e. pCa > 9 to pCa 4.7), as in Fig. 1. Specific force (i.e. maximum force per unit cross-sectional area) in type II fibres was on average ∼17% lower in fibres of Old compared to Young, whereas specific force in type I fibres did not differ significantly between the two age groups (Fig. 2). Two-way ANOVA showed that in both males and females specific force was reduced with age in type II fibres and not in type I fibres. The Ca^{2+} sensitivity of the type II fibres was also found to be lower in Old relative to Young (pCa_{50} ∼0.05 pCa units lower), but in type I fibres

Table 1. Ca^{2+} sensitivity of fibres from Young and Old subjects

	Type I fibres		Type II fibres	
	Young ($n = 26$, $N = 7$)	Old ($n = 46$, $N = 8$)	Young ($n = 21$, $N = 7$)	Old ($n = 25$, $N = 8$)
pCa_{50}	6.01 ± 0.04	5.99 ± 0.08	5.96 ± 0.04*	5.91 ± 0.09*#
h	4.4 ± 0.7	4.2 ± 0.8	5.2 ± 0.8*	5.4 ± 0.9*

Means ± SD of pCa_{50} and Hill coefficient (h) in type I and type II fibres. n denotes number of fibres and N the number of subjects. #Value in Old group is significantly different from the matching value in the Young group; *value for type II fibres is significantly different from that in type I fibres in same age group (Student's two-tailed t tests).

it was not significantly different between the age groups (Table 1). As expected, type II fibres showed a lower Ca^{2+} sensitivity (lower pCa_{50}) and steeper Hill coefficient (h) than type I fibres in both Old and Young (Table 1).

In order to examine whether the contractile properties had been affected by some reversible oxidative modification, the properties were tested both before and after strong reducing treatment with DTT (10 mM for 5 min) (e.g. Fig. 1). Such DTT treatment had no effect on either the specific force or the Ca^{2+} sensitivity in the type I fibres of either Young or Old subjects (Table 2). In the type II fibres, the DTT treatment also had little or no effect on specific force, but it did produce a small decrease in Ca^{2+} sensitivity (by ~0.015 pCa units) in the fibres of both Young and Old subjects (Table 2).

We have previously shown that treating skinned type II fibres from rats or humans successively with the sulphydryl-specific oxidant DTDP (100 μM, 5 min), and then reduced glutathione (GSH) (5 mM, 2 min), results in *S*-glutathionylation of the troponin I fast isoform (TnI_f), which induces a large increase in myofibrillar Ca^{2+} sensitivity (Lamb & Posterino, 2003; Mollica *et al.* 2012); this treatment regime induces the maximal increase in Ca^{2+} sensitivity in type II fibres but no significant increase in type I fibres. When this DTDP–GSH treatment was applied to type II fibres of the Young subjects in the present study, there was a large increase in Ca^{2+} sensitivity (mean (±SD) increase in pCa_{50} of 0.168 ± 0.015 pCa units), similar to that found previously (+0.171 ± 0.016 pCa units; Mollica *et al.* 2012), but in the type II fibres of the Old subjects the increase in Ca^{2+} sensitivity was ~20% smaller (0.136 ± 0.019 pCa units, $P < 0.05$), with no change in Hill coefficient in either case (Table 2). Figure 3 shows the mean Ca^{2+} sensitivity for the type II fibres of Young and Old subjects (continuous lines), and also the mean Ca^{2+} sensitivity prevailing after the DTDP–GSH treatment in the two cases (dashed lines); such a plot highlights the appreciable difference in force produced at the same free $[Ca^{2+}]$ in the Young and Old fibres and how this difference is increased following *S*-glutathionylation of the fibres by the DTDP–GSH treatment (>0.08 pCa units less sensitive in Old compared to Young), which could be expected to result in appreciable differences in muscle performance with age (see Discussion).

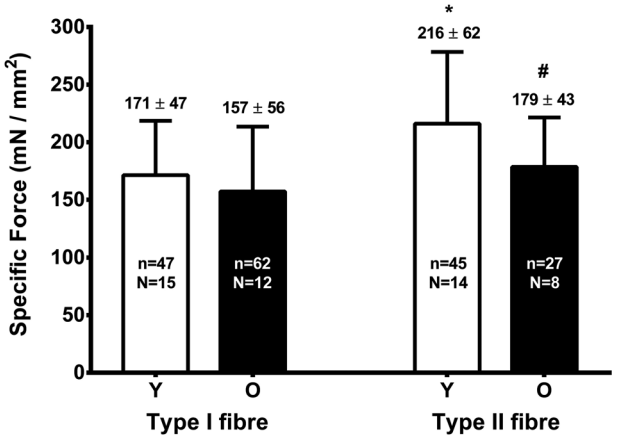

Figure 2. Specific force in type II fibres is significantly lower with age
Mean (+SD) of specific force in type I and type II fibres from Young (Y) and Old (O) subjects; specific force assessed by exposing skinned fibre to maximal activation solution. n denotes the number of fibres and N the number of subjects from which the biopsies were taken. *Significantly different from the type I fibre in the matching age group; #significantly different from Young group for matching fibre type (Student's two-tailed t test).

Ca^{2+} content measurements

The Ca^{2+} content of individual muscle fibres from the Old subjects was assayed with the SR in one of three different load states (Fig. 4) using the same fibre-lysing technique as used previously to assess the Ca^{2+} content in fibres of Young subjects (Lamboley *et al.* 2013). The Ca^{2+} content measurements in the fibres from the Old and Young cohorts were made during overlapping periods, using exactly the same stock solutions and procedures. The Ca^{2+} content assay involved pre-equilibrating the given skinned fibre for a short period with a carefully selected concentration of BAPTA, a high affinity Ca^{2+} chelator, and then lysing all intracellular compartments by immersing the fibre in an emulsion of paraffin oil and Triton X-100 (see Methods). This releases any Ca^{2+} stored within the fibre but keeps it within the fibre space, where it will elicit a force response if the free $[Ca^{2+}]$ reaches a sufficiently high

Table 2. Effects of DTT and *S*-glutathionylation treatments on contractile parameters

	Type I fibres		Type II fibres	
	Young ($n = 10$, $N = 3$)	Old ($n = 22$, $N = 3$)	Young ($n = 10$, $N = 3$)	Old ($n = 14$, $N = 3$)
ΔpCa_{50} DTT	0.000 ± 0.009	−0.002 ± 0.010	−0.014 ± 0.013*	−0.016 ± 0.013*
ΔMax (%) DTT	0.1 ± 2.7	0.1 ± 2.1	1.3 ± 1.2*	0.4 ± 2.0
ΔpCa_{50} Glut	—	—	0.168 ± 0.015*	0.136 ± 0.019*#
Δ*h* Glut	—	—	0.1 ± 0.4	−0.1 ± 0.3

Means ± SD of change (Δ) in pCa_{50} and maximum force (Max) following DTT treatment in type I and type II fibres, and change in pCa_{50} and Hill coefficient (*h*) following *S*-glutathionylation treatment (Glut) in type II fibres (as in Fig. 1). Values corrected for very small decline in maximum force and pCa_{50} occurring upon repeated examination of force–pCa staircase, as gauged by values obtained by repeating controls and with bracketing treatments with DTT. *n* denotes number of fibres and *N* the number of subjects. *Value is significantly different from zero; #value in Old group is significantly different from matching value in Young group (Student's two-tailed *t* tests).

level, making it possible to calculate the total amount of Ca^{2+} present from the size of the force response generated and the concentration of BAPTA present (see Methods, and Lamboley *et al.* 2013).

The Ca^{2+} content of fibres of Old subjects was assessed (i) in fibres containing their endogenous Ca^{2+} content (e.g. Fig. 4*A*), (ii) in fibres where the SR had been loaded to its maximal level by exposure to a Ca^{2+} load solution (pCa 6.7, 1 mM total EGTA) for 4 min (e.g. Fig. 4*B*), and (iii) in fibres where the SR had been depleted of Ca^{2+} by exposure to a 30 mM caffeine–low [Mg^{2+}] (pCa > 8.5, 1 mM free EGTA) solution for 1 min (e.g. Fig. 4*C*). It was found that for a given SR load state and fibre type, the Ca^{2+} content values in fibres of all Old subjects were very similar (see tight distributions in Fig. 5*A*). Similar to our previous findings in Young subjects (Lamboley *et al.* 2013), in the Old subjects the endogenous and maximal Ca^{2+} content in type II fibres were ∼10% and ∼20% higher, respectively, than the corresponding values in type I fibres (Table 3). Significantly, the endogenous and maximal Ca^{2+} contents in fibres of the Old subjects were ∼10% lower than the corresponding values in fibres of the Young subjects (Table 3). A further marked difference was that the amount of Ca^{2+} retained within a fibre after prolonged exposure to the caffeine–low [Mg^{2+}] 'full release solution' was considerably higher in the fibres of the Old subjects compared to those of the Young subjects (Table 3). In fact, following the SR Ca^{2+} depletion treatment, type II fibres of Old subjects on average still retained ∼0.17 mmol Ca^{2+} per litre fibre volume, which was more than 20% of their initial endogenous Ca^{2+} content. Taking these non-releasable amounts of Ca^{2+} into account, it was evident that the amount of Ca^{2+} that could be released from the SR in fibres of the Old subjects was substantially lower than that in the Young subjects (Fig. 5*B*), both in type I and type II fibres and when the SR was loaded either endogenously or maximally.

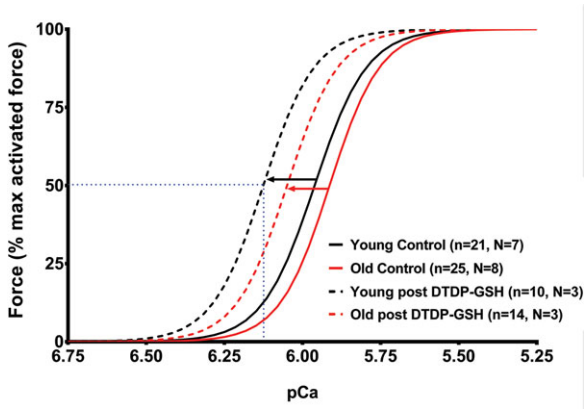

Figure 3. Type II muscle fibres from Old subjects are less sensitive to Ca^{2+} and also less responsive to *S*-glutathionylation
Hill curves plotted with a pCa_{50} and Hill coefficient value corresponding to the mean of the values found from individual fits to the force–pCa staircases (e.g. Fig. 1) for each type II muscle fibre from Young and Old subjects, before (control) and after *S*-glutathionylation treatment. Mean pCa_{50} (pCa at half-maximum force) (±SD) in control conditions was 5.96 ± 0.04 and 5.91 ± 0.09 in Young and Old subjects, respectively ($P < 0.05$), with average Hill coefficients (*h*) of 5.2 ± 0.8 and 5.4 ± 0.9, respectively (Table 1). *S*-Glutathionylation treatment produced a significantly smaller increase in Ca^{2+} sensitivity in fibres of Old subjects (red arrow, 0.136 ± 0.019 pCa units) than in fibres of Young subjects (black arrow, 0.168 ± 0.015 pCa units), with no significant change in *h*. Thus, following *S*-glutathionylation, the Ca^{2+} sensitivity of type II fibres in Old subjects (red dashed line) is substantially lower than in Young subjects (black dashed line), with the free [Ca^{2+}] producing 50% maximal force in Young fibres eliciting only ∼30% of maximal force in Old fibres (see dotted blue lines).

Comparative MHC and calsequestrin isoform content in muscle and fibres of Old subjects

Western blotting was used to assess whether the relative amounts of MHC and CSQ isoforms differed between the muscles of Old and Young subjects. Examination of individual fibres from muscles of Old subjects (data not shown) revealed that, similar to our previous findings in Young subjects (Lamboley *et al.* 2013), (i) the great

majority of fibres contained only the MHCI isoform or only MHCII isoforms (the latter being virtually all MHCIIa, rather than MHCIIx), and were hence classified as type I and type II, respectively, and (ii) all such type II fibres contained substantial amounts of CSQ1 and little or no CSQ2, and all type I fibres contained substantial amounts of CSQ2 and less CSQ1 than the type I fibres. Because of issues of measurement accuracy and sampling limitations with the single fibre data, the quantitative comparison between Old and Young subjects was undertaken by analysing muscle homogenate samples (e.g. Fig. 6). This analysis showed that the amount of MHCI present in muscle of the Old subjects on average was ∼124% of that present in muscle of the Young subjects, and the amount of MHCIIa present was ∼62% (Table 4). These values would be consistent with the ratio of type I to type II fibres being ∼50:50 in muscle of Young subjects and ∼65:35 in muscle of Old subjects, in accord with the distribution of fibre types found here in the single fibres dissected randomly from the muscles of the two age groups (type I:type II ratio of 53:47 in Young and 67:33 in Old, see top section of Results). It was apparent nevertheless that the MHC isoform proportions (i.e. fibre type proportions) differed considerably between different individuals, irrespective of age (e.g. see range of MHCI and MHCIIa blots at top of Fig. 6). It was also noted that the proportion of MHCI was particularly high in the muscle of all Old females examined (e.g. Fig. 6), but the sample size of subjects was not large enough to establish whether there was indeed any significant effect of sex.

Western blotting was also used to identify the presence of the MHCIIx isoform in homogenates of the

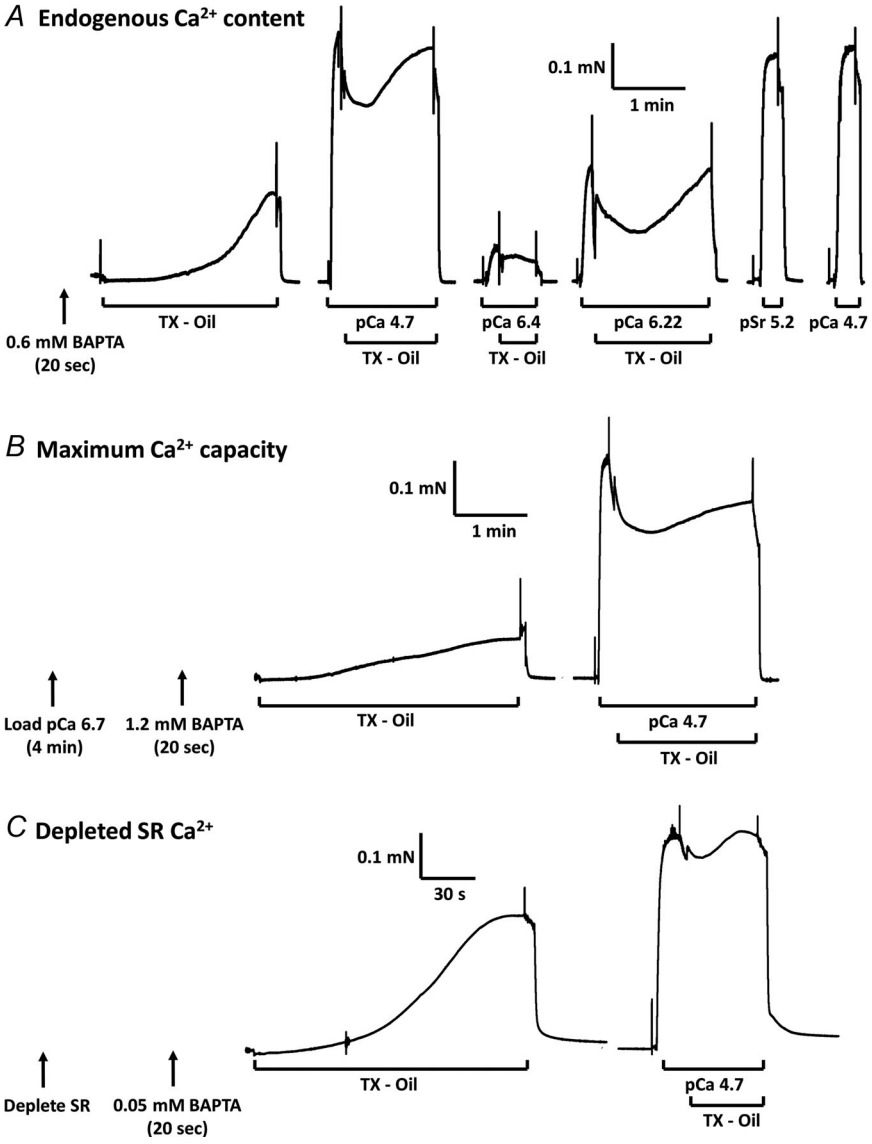

Figure 4. Ca^{2+} content assay in skinned fibre segments in three SR load states
All fibres from Old subjects. *A*, type I fibre containing endogenous Ca^{2+} content briefly equilibrated with 0.6 mM BAPTA and then lysed in Triton–paraffin oil emulsion (TX–Oil), producing ∼40% of maximal force. Fibre subsequently activated in solutions of various pCa and again transferred to TX–oil, showing TX–oil exposure did not appreciably alter myofibrillar Ca^{2+} sensitivity. *B*, another type I fibre, in which SR had been maximally loaded (by 4 min in pCa 6.7 load solution), needed to be equilibrated with 1.2 mM BAPTA to yield a finite non-maximal force response upon lysing. *C*, type II fibre segment exposed to caffeine–low Mg^{2+} solution (for 1 min) to try to fully deplete the SR of Ca^{2+}, still gave a large force response upon lysis in TX–oil.

different muscle samples. Using this methodology it was not possible to determine how much the total MHC was MHCIIx, but work of others has shown that in vastus lateralis muscle samples MHCIIx constitutes on average only ∼2–5% of the total MHC in Young and active Old subjects (D'Antona *et al.* 2007; Hvid *et al.* 2011). In the present study it was found that the relative amount of the MHCIIx isoform in the muscle (which was presumably present in fibres containing both MHCIIa and MHCIIx, see Methods) differed more than 10-fold between different subjects, both in the Young and the Old cohorts. On average, there was ∼1.3 times more MHCIIx in the muscle of the Old subjects relative to that in the Young subjects, but this difference was not statistically significant owing to the very large subject to subject differences. Interestingly, the relative amount of MHCIIx present in the muscle samples was found to be greater in muscle of subjects with a greater amount of MHCI (linear regression analysis, $r^2 = 0.389$, $P < 0.005$).

We also measured the relative amounts of calsequestrin present in the muscles (Fig. 6), in order to investigate whether the lower endogenous and maximal Ca^{2+} content values seen in muscle of the Old subjects (Table 3) could result from deficiency of these key SR Ca^{2+} storage proteins. The amounts of CSQ1 and CSQ2 protein in the muscle of the Old subjects were on average 1.07 times and 1.42 times the levels present in muscle of the Young subjects, respectively (Table 4). CSQ antibodies also detect a group of higher molecular weight proteins, referred to as 'CSQ-like proteins', which are also present in the SR in both skeletal and cardiac muscle (Cala *et al.* 1990; Culligan *et al.* 2002; Murphy *et al.* 2009*a*). These CSQ-like proteins were present in muscle of both the Young and Old subjects, but a striking difference was that the lowest of these protein bands, running at ∼105 kDa (Fig. 6, arrow), was present in ∼3- to 4-fold higher amounts in muscle of Old subjects (Table 4).

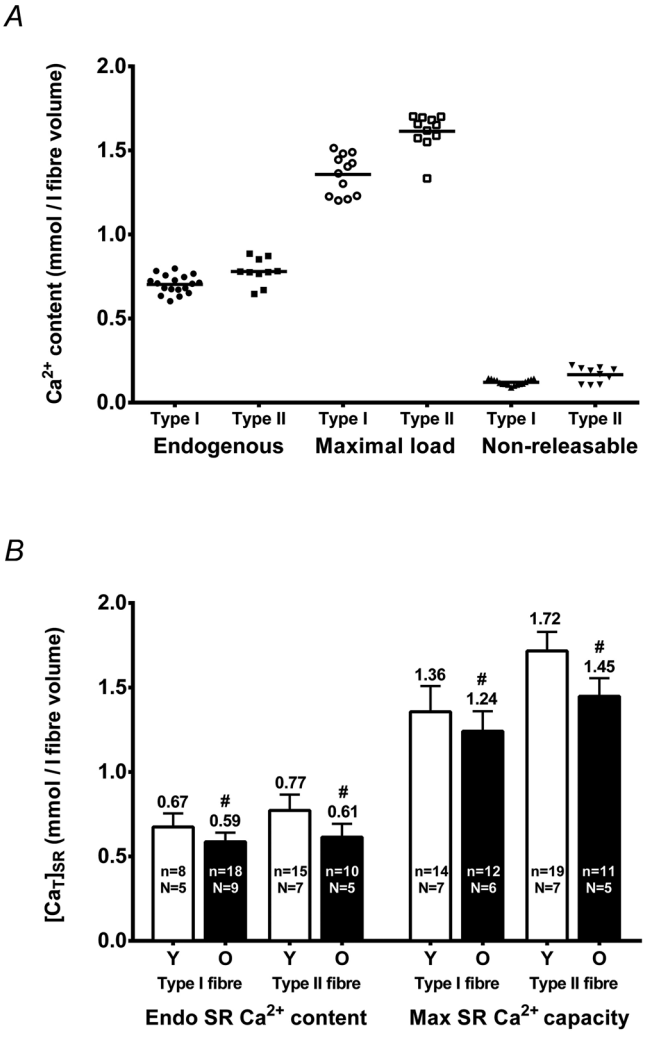

Figure 5. Total and releasable Ca^{2+} content is decreased in fibres of Old subjects
A, endogenous, maximal and non-releasable Ca^{2+} content of individual type I and II fibres from Old subjects, assayed as in Fig. 4. Horizontal lines indicate mean levels. *B*, mean (+SD) amount of releasable Ca^{2+} in SR of fibres of Young and Old subjects at endogenous and maximal load; values derived by subtracting corresponding mean amount of non-releasable Ca^{2+}. Original data for Young subjects presented previously (Lamboley *et al.* 2013). #Value in Old (O) is significantly different from that in Young (Y) (Student's two-tailed *t* test, $P < 0.05$).

Myosin density and myosin to actin ratio

Finally, in order to investigate whether there was any change in the myosin density with age, muscle homogenates were treated with Triton X-100 and spun to separate the cytoskeletal proteins (pellet) from the cytosolic and membrane-associated proteins (see Methods), and then the cytoskeletal proteins were separated on a Stain-Free gel, allowing identification and quantification of the myosin and actin bands. It was found that the amount of myosin relative to total protein in these pellet samples was not significantly different between muscle of Old and Young subjects (0.24 ± 0.02 ($n = 15$) and 0.23 ± 0.02 ($n = 11$), respectively, $P = 0.58$, Student's two-tailed *t* test). Furthermore, the density of the myosin band relative to the actin band in the muscle samples was also not significantly different between the Old and Young subjects (0.71 ± 0.06 ($n = 15$) and 0.67 ± 0.06 ($n = 11$), respectively, $P = 0.17$, Student's two-tailed *t* test).

Discussion

This study, using mechanically skinned fibres obtained from fresh biopsies of vastus lateralis muscle, found a number of significant differences between the muscle fibres of the Old and Young subjects, despite the Old

Table 3. Summary of Ca^{2+} content measurements for various SR load conditions

	Type I fibre		Type II fibre	
	Young	Old	Young	Old
$[Ca_T]_{endo}$ (mmol l^{-1})	0.76 ± 0.08 ($n = 8$; $N = 5$)	$0.70 \pm 0.05^{\#}$ ($n = 18$; $N = 9$)	$0.85 \pm 0.09^{*}$ ($n = 15$; $N = 7$)	$0.78 \pm 0.08^{*\#}$ ($n = 10$; $N = 5$)
$[Ca_T]_{max}$ (mmol l^{-1})	1.44 ± 0.15 ($n = 14$; $N = 7$)	1.36 ± 0.12 ($n = 12$; $N = 6$)	$1.79 \pm 0.11^{*}$ ($n = 19$; $N = 7$)	$1.61 \pm 0.11^{*\#}$ ($n = 11$; $N = 5$)
$[Ca_T]_{left}$ (mmol l^{-1})	0.08 ± 0.02 ($n = 8$; $N = 3$)	$0.12 \pm 0.01^{\#}$ ($n = 14$; $N = 7$)	$<0.073^{*}$ ($n = 11$; $N = 5$)	$0.17 \pm 0.05^{*\#}$ ($n = 10$; $N = 4$)

Values are means $\pm$ SD of total Ca^{2+} content ($[Ca_T]$) in type I and type II fibres from Young and Old subjects for three SR load states: endogenous content (subscript 'endo'), maximum capacity (subscript 'max') and fully depleted SR (subscript 'left'), assayed as in Fig. 4. Data for Young subjects presented previously (Lamboley *et al.* 2013). *n* denotes the number of fibres examined and *N* the number of subjects from which biopsies were taken. $^{\#}$Value in Old group is significantly different from matching value in Young group; *value in type II fibres is significantly different from that in type I fibres in same age group (Student's two-tailed *t* tests).

Table 4. Relative amounts of MHC and CSQ isoforms in muscle of Old and Young subjects

	Old subjects ($N = 11$)	Young subjects ($N = 10$)
MHCI	1.24 ± 0.46	1.00 ± 0.25
MHCIIa	$0.62 \pm 0.36^{*}$	1.00 ± 0.22
CSQ1	1.07 ± 0.33	1.00 ± 0.23
CSQ2	$1.42 \pm 0.36^{*}$	1.00 ± 0.25
CSQ-like ~105 kDa band	$3.64 \pm 3.25^{*}$	1.00 ± 1.04
CSQ-like (sum of all bands)	1.13 ± 0.50	1.00 ± 0.41

Values are means $\pm$ SD of relative amount of indicated isoform present in muscle homogenates of Old and Young subjects. Samples from all 21 subjects run on each gel, together with mixed homogenate samples for verifying signal detection range (see Fig. 6). For each gel, the intensity of the relevant band was first normalized by the amount of muscle sample loaded in that lane (determined from relative lane intensity on Stain-Free image), and then was expressed relative to the average of the values found in the Young samples. Values given for each protein are the average of measurements made on two to four gels. *Value in Old subjects is significantly different from that in Young subjects (Student's *t* test; Wilcoxin Mann–Whitney rank test used with CSQ-like band data, which was not normally distributed).

subjects maintaining an active lifestyle. Firstly, the proportion of type II fibres and MHCIIa (see Results and Table 4) was substantially lower on average in the muscle of the Old subjects, similar to that reported in a number of other studies (Poggi *et al.* 1987; Marzani *et al.* 2005; Staunton *et al.* 2012). It was further found that the type II fibres of the Old subjects produced ~17% lower specific force (Fig. 2) and also had decreased Ca^{2+} sensitivity (Table 1). A number of previous studies, using fibres that were chemically skinned and stored, have reported that the specific force was lower in either or both type I and type II fibres in Old subjects compared to Young subjects (Larsson *et al.* 1997; Frontera *et al.* 2000; D'Antona *et al.* 2003; Yu *et al.* 2007; Hvid *et al.* 2013*a*), with the effects being greater in Old subjects with reduced activity (Larsson *et al.* 1997; D'Antona *et al.* 2007). With regard to Ca^{2+} sensitivity, the only previous studies comparing muscle from Old and Young human subjects were conducted using chemically skinned vastus lateralis fibres and, interestingly, quite similar results were found to those of this study, with the mean pCa_{50} in the type II fibres being 0.08 and 0.09 pCa units lower in the Old subjects relative to the Young subjects (Hvid *et al.* 2011, 2013*b*), though in both those studies this difference did not reach the significance level.

The differences in contractile properties in type II fibres between Old and Young subjects were not attributable to reversible oxidative modification, because strong reducing treatment with DTT did not produce any significant increase in specific force in the Old fibres, and furthermore led to a similar (small) decrease in Ca^{2+} sensitivity (~0.015 pCa units) in both Old and Young fibres (Table 2). This decrease in Ca^{2+} sensitivity with DTT treatment occurred only in the type II fibres, and not in the type I fibres (Table 2), most likely reflecting that even in subjects at rest there is a low but finite level of *S*-glutathionylation of TnI_f in the type II fibres, as was directly observed previously in other rested subjects (see Fig. 11 in Mollica *et al.* 2012). This is quite likely the mechanistic basis of observations made previously in mouse fast-twitch fibres

in vitro where DTT treatment was observed to cause a decrease in contractile apparatus Ca^{2+} sensitivity in unstimulated fibres, which was taken to indicate that the fibres were oxidized to some extent even at rest and that this increased the Ca^{2+} sensitivity above the level pertaining in a fully reduced state (Andrade *et al.* 1998).

A further important observation here was that maximal increase in Ca^{2+} sensitivity occurring with *S*-glutathionylation treatment of TnI_f in the type II fibres was ~20% less in the fibres of Old subjects (mean increase: 0.136 and 0.168 pCa units in Old and Young fibres, respectively, Table 2). The reason for this difference is not known, though it was clearly not due to a difference in the resting level of TnI_f *S*-glutathionylation, given that the DTDP–GSH treatment was applied only after first fully reducing the fibres with DTT (see Fig. 1). One possibility is that the type II fibres in the Old subjects had already undergone some level of *irreversible* oxidative change, which either directly prevented *S*-glutathionylation of the critical cysteine residue on a portion of the TnI_f molecules or decreased the extent of the sensitivity increase produced by *S*-glutathionylation. This possibility is suggested by our previous finding that strong oxidative treatment of type II fibres irreversibly blocks the ability of the *S*-glutathionylation treatment to increase Ca^{2+} sensitivity (Murphy *et al.* 2008) and by our unpublished observation that the damaging effects of the strong oxidative treatment can be largely prevented if fibres are given the *S*-glutathionylation treatment beforehand (G. D. Lamb, unpublished observations). The possibility that the fibres in the Old subjects had undergone some level of irreversible oxidative change would also be consistent with previous reports of other measures of oxidative damage occurring in aged humans (Fano *et al.* 2001; Fulle *et al.* 2004) and rodents (Prochniewicz *et al.* 2005, 2007; Jackson & McArdle, 2011; McDonagh *et al.* 2014; Tohma *et al.* 2014). Importantly too, it would also account for the decreases in specific force and Ca^{2+} sensitivity seen here in the same fibres (Fig. 2 and Table 1). Strong oxidative treatment is known to cause irreversible decreases in specific force and Ca^{2+} sensitivity in isolated fibres and muscle (see Introduction), and oxidant-linked reductions in specific force, with or without an accompanying decrease in Ca^{2+} sensitivity, have also been found to occur in skeletal and diaphragm muscle in many situations, including with sepsis or endotoxin application (Supinski *et al.* 2000; Callahan *et al.* 2001*a*), with TNFα or sphingomyelinase treatment (Hardin *et al.* 2008; Ferreira *et al.* 2012), and with hypoxia or hyperoxia (Ottenheijm *et al.* 2006; Dutka *et al.* 2012). Proteins are constantly turning over in muscle, so the observed accumulation of oxidized proteins in muscle in ageing and other conditions implies that there must be a continual level of oxidative modification occurring that effectively keeps pace with protein turnover.

The exact processes underlying these putative irreversible oxidative changes are uncertain, but quite possibly involve the progressive transition of cysteine residues through reversible oxidized states, such as *S*-nitrosylation (RSNO) or sulphenation (RSOH), to poorly reversible or irreversible states such as sulphination (RSO_2H) and sulphonation (RSO_3H) (Halliwell & Gutteridge, 2007; Lamb & Westerblad, 2011), or alternatively could involve oxidative changes to methionine residues (Stadtman *et al.* 2005). The fact that the decreases in specific force and Ca^{2+} sensitivity occurred only in the type II fibres and not in type I fibres is quite possibly because the levels of reduced GSH, the major cellular anti-oxidant, as well as GSH peroxidase, GSSG reductase and catalase are all several times higher in type I fibres than in type II fibres (at least in rat) (Ji *et al.* 1992). This would probably mean that reactive

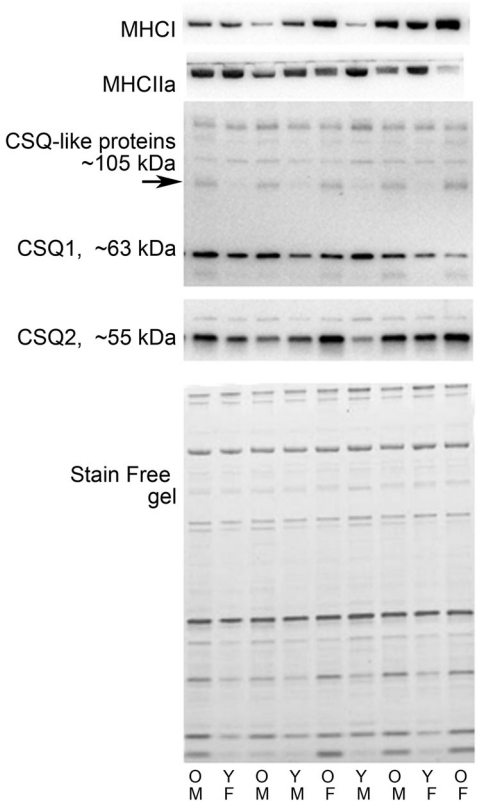

Figure 6. MHC and CSQ isoforms in muscle homogenates of Young and Old subjects
Western blots for MHCI and MHCIIa, and CSQ1 and CSQ2, in muscle of 9 subjects. CSQ antibody also labels a group of higher molecular weight proteins, collectively referred to as CSQ-like proteins; arrow points to the lowest of these, running at ~105 kDa, which was increased in muscle of Old subjects. Labels identify subjects as Old (O) or Young (Y), and male (M) or female (F). Lowest panel: pre-transfer Stain-Free gel for the CSQ1 and CSQ2 Western blots, showing relative amount of protein loaded in each lane. MHCI and MHCIIa blots shown are for the same amounts of samples as those run on the gel shown.

oxygen species are better counteracted in type I fibres, presumably decreasing the propensity of target sites to progress through to irreversibly oxidized states.

Irrespective of whether or not oxidative changes are responsible, the observed decreases in specific force and Ca^{2+} sensitivity, and decreased responsiveness to *S*-glutathionylation, in the type II fibres in Old subjects must inevitably have appreciable deleterious effects on overall muscle performance in those subjects. Firstly, even if there were no net loss of muscle tissue, the decrease in specific force in the type II fibres, and the increased proportion of type I fibres, would mean that the maximum force production of the muscles in those subjects would be decreased. Secondly, the lower contractile apparatus Ca^{2+} sensitivity would mean that the muscle fibres produce less force than fibres of Young subjects at the same intracellular free [Ca^{2+}] (Fig. 3), and this effect would be even more marked during heavy exercise, when the decreased *S*-glutathionylation effect would mean that the fibres in the Old subjects were less able to counter the deleterious effects on Ca^{2+} sensitivity of the build-up of inorganic phosphate and other metabolites (Allen *et al.* 2008; Mollica *et al.* 2012).

Calcium content of muscle fibres

A further major finding was that, in both type I and type II fibres, both the endogenous and maximal releasable SR Ca^{2+} content were ∼15% lower in muscle of Old subjects compared to Young subjects (Fig. 5*B*). To our knowledge there has been no previous measurement of the SR Ca^{2+} content in muscle of aged human subjects. Delbono *et al.* (1995) reported that the peak of the intracellular Ca^{2+} transient to voltage steps in putative fast-twitch fibres from vastus lateralis muscle was ∼30% lower in fibres of old subjects than in young subjects. That study also concluded that the difference in peak Ca^{2+} release was not due to 'SR calcium depletion' because the peak of the Ca^{2+} transient in fibres of both old and young subjects could be increased ∼20% by having 0.5 mM caffeine present during the voltage steps. This, however, only indicates that the SR still contained some finite level of Ca^{2+} at the point of peak release in the control situation with no caffeine, and provides no information as to whether the endogenous SR Ca^{2+} content of the fibres was any different between the old and young subjects. The findings of the present study point to the possibility that the Ca^{2+} transient was smaller in the fibres of the Old subjects because less Ca^{2+} was stored in the SR, which would have lowered the driving force for Ca^{2+} efflux. Our group has previously shown that the amount of Ca^{2+} released by action potential stimulation in mammalian muscle fibres remains constant if the SR is loaded above its normal endogenous level, but decreases markedly if the SR is loaded at less than its normal endogenous level (see Fig. 10*C* in Posterino & Lamb, 2003).

The lower endogenous and maximal SR Ca^{2+} content in the fibres of the Old subjects was not attributable to there being less calsequestrin (Table 4). In fact, taking into account the increased proportion of type I fibres in the muscle of the Old subjects (see Results), and the relative amounts of CSQ1 and CSQ2 in type I and type II fibres (Lamboley *et al.* 2013), the data indicate that the levels of both CSQ1 and CSQ2 were ∼20% *higher* in fibres of Old compared to Young subjects. It is possible that the calsequestrin in the Old subjects was in some way altered (e.g. oxidized, phosphorylated etc.) and did not bind Ca^{2+} with the same efficacy as that in Young muscle, but there are no data or information to suggest this. We did observe, however, that there was a marked increase in the amount of a ∼105 kDa CSQ-like protein in the muscle of the Old subjects (Fig. 6 and Table 4). The function of the CSQ-like proteins remains unknown (Cala *et al.* 1990; Culligan *et al.* 2002), though they appear not to bind appreciable Ca^{2+} (Murphy *et al.* 2009*a*). It has been reported previously that the density of the CSQ-like proteins increases with age in rat muscle (Chevessier *et al.* 2004), but no such increase was seen in another study on human muscle, though only a relatively small number of subjects were examined (Ryan *et al.* 2003). It is possible that the 105 kDa protein CSQ-like protein is a dimer or other cross-linked form of calsequestrin (Cala *et al.* 1990), or that the increase in its amount in some way interferes with Ca^{2+} binding to calsequestrin. We believe, however, that the primary reason for the reduced level of SR Ca^{2+} in the muscle fibres of the Old subjects is that there is increased leakage of Ca^{2+} out of the SR (authors' unpublished observations), similar to that reported in muscle fibres of old mice (Andersson *et al.* 2011).

Another potentially important finding was that in the muscle of Old subjects significantly more of the total Ca^{2+} present within the muscle fibres was not released upon prolonged exposure to the caffeine–low [Mg^{2+}] 'full release solution' (Table 3), with almost 20% of the endogenous Ca^{2+} remaining unreleased in the case of type II fibres. It is possible that this residual Ca^{2+} was present in the mitochondria or some other membranous compartment, or instead in isolated/fragmented regions of SR, such as those reported in muscle of old mice (Weisleder *et al.* 2006) or in 'disassembling' triadic junctions seen in muscle of old humans (Boncompagni *et al.* 2006). Interestingly, our finding appears quite analogous to that of a recent study in mouse muscle fibres where it was found that at the end of prolonged voltage steps significantly more Ca^{2+} remained within the SR in the fibres of old mice compared to young mice (Wang *et al.* 2012). Such a non-releasing pool of Ca^{2+} could not contribute in any direct way to force production by the muscle fibre, irrespective of whether it was located specifically in pockets of isolated SR or instead was distributed uniformly throughout the whole SR.

Conclusion

This study identified a number of important functional differences in the skeletal muscle fibres of Old subjects relative to Young subjects, involving both the contractile apparatus and the storage and release of intracellular Ca^{2+}. Type II fibres of Old subjects displayed decreased specific force and myofibrillar Ca^{2+} sensitivity and also a smaller increase in Ca^{2+} sensitivity with *S*-glutathionylation treatment, all features that could arise through oxidant-induced damage. In addition, in both type I and type II fibres, the amount of releasable Ca^{2+} stored in the SR, and the maximal SR storage capacity, were significantly lower in muscle of the Old subjects, which could not be attributed to differences in calsequestrin levels. Furthermore, a significantly greater amount of Ca^{2+} remained in the fibres of the Old subjects following prolonged exposure to a potent caffeine–low $[Mg^{2+}]$ releasing solution, which could be indicative of differences in Ca^{2+} compartmentalization or movement in the muscle fibres of the Old subjects. Singly, and particularly together, these differences in the muscle fibre properties in the Old adults could be expected to have adverse effects on muscle strength and performance, and may be significant contributory factors to muscle weakness seen in old age.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

Subjects were tested and muscle biopsies performed at Victoria University, and biochemical and physiological measurements on skinned fibres and homogenates made at La Trobe University. C.R.L., V.L.W. and M.J.M. were responsible for selection, care and testing of subjects, and obtaining muscle biopsies. Skinned fibre experiments were designed and analysed by G.D.L. and C.R.L. and carried out by C.R.L. and T.L.D., and R.M.M. and G.D.L. were responsible for Western blotting procedures and analyses, with the assistance of V.L.W. C.R.L. and G.D.L. drafted the manuscript. All authors were involved in the conception of the project and have reviewed the final version of the submitted manuscript.

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