Ca^{2+} leakage out of the sarcoplasmic reticulum is increased in type I skeletal muscle fibres in aged humans

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Running Head: Increased SR Ca^{2+} leakage in type I fibres with age

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Key Points Summary

- The amount of Ca^{2+} stored in the sarcoplasmic reticulum (SR) of muscle fibres is decreased in aged individuals, and an important question is whether this results from increased Ca^{2+} leakage out through the Ca^{2+} release channels (ryanodine receptors, RyRs).
- This study examined the effects of blocking the RyRs with Mg^{2+}, or applying a strong reducing treatment, on net Ca^{2+} accumulation by the SR in skinned muscle fibres from Old (~70 yrs) and Young (~24 yrs) adults.
- Raising cytoplasmic [Mg^{2+}] and reducing treatment both increased net SR Ca^{2+} accumulation in type I fibres of Old subjects relative to that in Young.
- The densities of RyRs and dihydropyridine receptors were not significantly changed in the muscle of Old subjects.
- These findings indicate that oxidative modification of the RyRs causes increased Ca^{2+} leakage from the SR in muscle fibres in Old subjects, which likely deleteriously affects normal muscle function both directly and indirectly.

Abstract

This study examined whether the lower Ca^{2+} storage levels in the sarcoplasmic reticulum (SR) in *vastus lateralis* muscle fibres in Old (70 ± 4 years) relative to Young (24 ± 4 years) human subjects is the result of increased leakage of Ca^{2+} out of the SR through the Ca^{2+} release channels/ryanodine receptors (RyRs) and due to oxidative modification of the RyRs. SR Ca^{2+} accumulation in mechanically skinned muscle fibres was examined in the presence of 1, 3 or 10 mM cytoplasmic Mg^{2+}, as raising [Mg^{2+}] strongly inhibits Ca^{2+} efflux through the RyRs. In type I fibres of Old subjects, SR Ca^{2+} accumulation in the presence of 1 mM Mg^{2+} approached saturation at shorter loading times than in Young subjects, consistent with Ca^{2+} leakage limiting net uptake, and raising [Mg^{2+}] to 10 mM in such fibres increased maximal SR Ca^{2+} accumulation. No such differences were seen in type II fibres. Treatment with dithiothreitol (10 mM for 5 min), a strong reducing agent, also increased maximal SR Ca^{2+} accumulation at 1 mM Mg^{2+} in type I fibres of Old subjects but not in other fibres. The densities of dihydropyridine receptors and RyRs were not significantly different in muscle of Old relative to Young subjects. These findings indicate that Ca^{2+} leakage from the SR is increased in type I fibres in Old subjects by reversible oxidative modification of the RyRs; this increased SR Ca^{2+} leak would be expected to have both direct and indirect deleterious effects on Ca^{2+} movements and muscle function.

Abbreviations List-

CaEGTA, calcium bound to EGTA; CSQ, calsequestrin; DHPR, dihydropyridine receptor; HDTA, hexa-methylene-diamine-tetraacetate; *h*, Hill coefficient; MHC, myosin heavy chain; n, number of fibres; N, number of subjects; pCa, $-\log_{10}[Ca^{2+}]$; RyR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase.

Introduction

Skeletal muscle performance declines in old age in humans and other species, even in active individuals (Ballak *et al.*, 2014; Miller *et al.*, 2014). This decline in performance is due both to loss of total muscle mass and to changes within the muscle fibres themselves. Force production at the single fibre level involves a complex sequence of events starting with the spread of an action potential along the muscle fibre and into the transverse tubular (t-) system, where it activates the dihydropyridine receptors (DHPRs), which by some protein-protein interaction open the ryanodine receptor (RyR)/Ca^{2+} release channels in the terminal cisternae of the adjacent sarcoplasmic reticulum (SR), allowing Ca^{2+} stored in the SR to move into the cytoplasm and activate the contractile proteins (Bottinelli & Reggiani, 2000; Rebbeck *et al.*, 2014). The decline in single muscle fibre performance in old age may be the result of some level of dysfunction in several different steps in this sequence, including disrupted coupling between the DHPRs and RyRs (Delbono *et al.*, 1995; Wang *et al.*, 2000; Boncompagni *et al.*, 2006), reduced storage and release of SR Ca^{2+} (Jimenez-Moreno *et al.*, 2008; Andersson *et al.*, 2011; Lamboley *et al.*, 2015), and reduction in both the Ca^{2+}-sensitivity and maximal force production by the contractile apparatus (D'Antona *et al.*, 2003; Yu *et al.*, 2007; Hvid *et al.*, 2011; Lamboley *et al.*, 2015).

It has been reported that in the skeletal muscle of aged (24 month old) mice there is increased 'leakage' of Ca^{2+} out of the SR into the cytoplasm through the RyRs, stemming from a self-reinforcing cycle in which oxidation and/or nitrosylation of the RyRs increases Ca^{2+} efflux from the SR, resulting in increased Ca^{2+} uptake by mitochondria, which in turn leads to increased production of reactive oxygen species and further oxidation of the RyRs (Andersson *et al.*, 2011; Umanskaya *et al.*, 2014). This Ca^{2+} leakage through the RyRs causes a decreased level of Ca^{2+} accumulation in the SR, and could be prevented by overexpression of catalase in the mitochondria or by in-vitro treatment of the muscle fibres with the reducing agent dithiothreitol (DTT) (Umanskaya *et al.*, 2014). Such Ca^{2+} leakage might contribute to decreased muscle performance in several ways, both directly by decreasing the amount of stored Ca^{2+} available for release, and indirectly by triggering

Ca^{2+}-dependent disruption of the triad junction (Lamb *et al.*, 1995; Murphy *et al.*, 2013) or oxidation-dependent dysfunction of the contractile apparatus or Ca^{2+} release (Reid & Durham, 2002; Prochniewicz *et al.*, 2007; Murphy *et al.*, 2008; Dutka *et al.*, 2011) or energy supply.

In the present study we examined whether there is increased Ca^{2+} leakage through the RyRs in the skeletal muscle fibres of aged humans, which could help account for the reduced SR Ca^{2+} content in such fibres and possibly also for the observed contractile apparatus dysfunction (Lamboley *et al.*, 2015). This was investigated by examining the SR Ca^{2+} accumulation properties in mechanically skinned *vastus lateralis* muscle fibres from Old (70 ± 4 years) and Young (24 ± 4 years) subjects, and in particular the ability of raised cytoplasmic [Mg^{2+}] to increase SR Ca^{2+} accumulation. Ca^{2+} efflux through the RyRs is strongly inhibited by cytoplasmic Mg^{2+} (Meissner *et al.*, 1986; Laver *et al.*, 2004). In the presence of 1 mM Mg^{2+}, the normal cytoplasmic concentration, the resting Ca^{2+} efflux rate through the RyRs is already very low (< 0.05% of the maximum efflux rate) in both SR vesicles (Meissner *et al.*, 1986) and mechanically skinned muscle fibres (Lamb *et al.*, 2001), and raising the [Mg^{2+}] from 1 to 3 mM decreases Ca^{2+} efflux by a further 5 fold in heavily Ca^{2+} loaded SR vesicles (Meissner *et al.*, 1986) and strongly inhibits the ability of caffeine and Ca^{2+} to induce Ca^{2+} release in skinned fibres and single RyRs (Lamb *et al.*, 2001; Laver *et al.*, 2004). We hypothesized that SR Ca^{2+} leakage is elevated in muscle fibres in Old subjects and would be decreased by raising the cytoplasmic [Mg^{2+}] from 1 mM to 10 mM, thereby producing an appreciable net increase in Ca^{2+} accumulation in fibres of Old subjects but not Young subjects. We further hypothesized that the increased SR Ca^{2+} leakage was the result of oxidation and/or nitrosylation of the RyRs and would be decreased by treating the fibres with DTT, as observed in muscle fibres of aged mice (Umanskaya *et al.*, 2014).

Finally, we also examined whether there was any decrease in the density of the DHPRs in the muscle of Old human subjects, as decreased DHPR density has been observed in muscle of aged mice (Renganathan *et al.*, 1997) and proposed to be the cause of the reduced SR Ca^{2+} release seen in both mouse (Wang *et al.*, 2000) and human muscle fibres (Delbono *et al.*, 1995). A decrease in the ratio of DHPRs to RyRs might result in less efficient activation of the RyRs during action potential stimulation, and also cause increased Ca^{2+} leakage through the RyRs in resting conditions owing to disruption of the ability of the DHPRs to exert some level of resting inhibition on the RyRs (Zhou *et al.*, 2006; Eltit *et al.*, 2011; Robin & Allard, 2012). We hypothesized that the density of the DHPRs, but not the RyRs, would be lower in muscle fibres of Old subjects relative to that in Young subjects.

Methods

Participants

This study was approved by the Human Research Ethics Committees of Victoria University and La Trobe University, and conformed to the Declaration of Helsinki. The muscle tissue used here was obtained as part of the study described in detail in Lamboley *et al*. (2015), supplemented with tissue from three additional Young subjects. A total of nineteen Young (12 males and 7 females) and nineteen Old adult subjects (13 males and 6 females) gave signed informed consent after reading all information. The mean age (± SD) was 70 ± 4 years in the Old group and 24 ± 3 years in the Young group. All participants were healthy and recreationally active but were not specifically trained in any sport and the physical activity level was similar between the Old and Young cohorts, as described in detail in Lamboley *et al*. (2015). The muscle tissue was obtained from a thigh muscle biopsy in rested conditions by an experienced medical practitioner. 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. 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. Physiological measurements were made on single fibres from a total of 13 Young (9 males and 4 females) and 14 Old subjects (10 males and 4 females), all of which were fibre-typed by western blotting (see below). Western blotting of muscle homogenates was performed on tissue from a total of 10 Young (4 males and 6 females) and 11 Old subjects (7 males and 4 females), using the same homogenate tissue as that reported in Lamboley *et al*. (2015), with fibres from 4 of these Young subjects and 6 of these Old subjects being used in the physiological fibre measurements.

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 under the paraffin oil as described previously (Murphy *et al.*, 2009; Lamboley *et al.*, 2013). 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, NSW, 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+}. The SR was reloaded with Ca^{2+} by exposing the fibre for a set time (10 to 180 s) to a load solution similar to the standard K-HDTA solution but with 1 mM total EGTA and the pCa buffered at 6.7 (i.e. 0.5 mM CaEGTA and 0.5 mM free EGTA), and with the standard 1 mM free Mg^{2+} or with 12 or 22.7 mM total Mg^{2+} to give 3 and 10 mM free Mg^{2+}, respectively (see (Lamb & Stephenson, 1991)). Maximum force was determined in a solution similar to HDTA solution but with 50 mM EGTA and 49.5 mM Ca^{2+} (pCa 4.7) and 8.1 mM total Mg^{2+} to maintain the free [Mg^{2+}] at 1 mM.

Ca^{2+} uptake and release protocol.

Ca^{2+} accumulation by the SR in the skinned fibres was examined similarly to that described previously (Lamboley *et al.*, 2013). As the muscle fibres were kept and skinned under paraffin oil, the SR initially retained its endogenous level of Ca^{2+}. Each skinned fibre segment was first bathed for 2 min in the standard K-HDTA solution to wash out all diffusible Ca^{2+} buffers present endogenously in the cytoplasm. The SR was then depleted of all of its releasable Ca^{2+} by exposing the fibre to the full release solution (see above). The fibre was then washed for 1min in standard K-HDTA solution (with 0.5 mM free EGTA present to prevent any Ca2+ reuptake), and subjected to repeated load–release cycles as follows:

Step 1: Load SR for a set time (10–180 s) in a load solution (pCa 6.7 with 0.5 mM CaEGTA and 0.5 mM free EGTA) with a free [Mg^{2+}] of 1, 3 or 10 mM as indicated.

Step 2: Pre-equilibrate skinned fibre for 15 s in standard K-HDTA solution with 0.5 mM EGTA.

Step 3: Empty SR of all releasable Ca^{2+} by exposing fibre for 1 min to full release solution.

Step 4: Wash skinned fibre for 1 min in standard K-HDTA solution with 0.5 mM EGTA.

The time integral (area) of the force response to the initial exposure to the full release solution was indicative of the endogenous SR Ca^{2+} content initially present in the fibre, and the responses on subsequent load-release cycles indicated the SR content for the respective load time (e.g. see Fig. 1). The relationship between the time integral of the force response and load time was well fitted by an exponential fit approaching saturation after ~180 s (Fig. 2). The parameters were

adjusted to take into account the amount of SR Ca^{2+} that had to be released to elicit any detectable force in the presence of 0.5 mM EGTA, determined by back-extrapolation of the response area–load time curve to zero load time (see Fig. 4 *C*&D in Lamboley *et al*., 2013).

Western blotting

Whole muscle homogenates from Young (n=10) and Old (n=11) subjects were those used previously (Lamboley *et al.*, 2015). Single fibres were dissected from tissue under paraffin oil from a subset of those individuals, and placed directly into 10 µl of 1x solubilizing buffer (SB) which contained 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 v/v) with double distilled water, as previously described (Murphy *et al.*, 2011; Lamboley *et al.*, 2013). Fibres were stored at -80ºC until analyzed by western blotting. For both whole muscle homogenates or isolated individual fibres, a 4-5 point calibration curve consisting of the same mixed muscle homogenate were loaded onto each gel, as previously described (Lamboley *et al.*, 2013; Murphy & Lamb, 2013). Total protein was separated on 4-12% Bis-Tris Criterion gels (Bio-Rad, Hercules, CA, USA) for 1 h at 200V with a MOPS running buffer (1 M MOPS, 1 M Tris Base, 69 mM SDS and 20.5 mM EDTA), with 150 µl beta-mercaptoethanol added to the top chamber of the electrophoretic tank after the samples were loaded in the gel. Protein was wet-transferred onto nitrocellulose membrane (100V for 30 min). Following transfer, the gel was stained with Biosafe coomassie stain (Bio-Rad), de-stained with water overnight and an image collected using a Chemidoc MP with ImageLab software (both BioRad). Membranes were treated with Miser antibody extender solution (ThermoFisher Scientific, Scoresby, Vic, Australia), and then blocked in 5% skim milk in Tris buffered saline with Tween (TBST) for 1-2 h at room temperature (RT). Membranes were exposed to primary antibodies overnight at 4°C and 2 h at RT. Antibodies used and their dilutions were: myosin heavy chain IIa (MHCIIa, mouse IgG, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA: A4.74, 1 in 400), myosin heavy chain 1 (MHCI, mouse IgM, DSHB: A4.840,1 in 100); ryanodine receptor 1 (RyR1, mouse, DSHB: 34C, 1 in 100), dihydropyridine receptor (DHPR, mouse, DSHB: IIID5EI, 1 in 400), SERCA1 (mouse DSHB: CaF2-5D2, 1 in 1000), SERCA2a (rabbit, Badrilla, Leeds, UK, 1 in 5000). The horseradish peroxidase (HRP) secondary antibodies used were goat anti-mouse HRP (ThermoFisher Scientific: PIE31430, 1 in 20,000), goat anti-rabbit HRP (ThermoFisher Scientific: PIE31460, 1 in 60,000) or goat anti-mouse IgM (Santa Cruz Biotechnology, Dallas, Texas, USA: sc-2064, 1 in 20,000). Specific immunoreactive bands were visualized using West Femto chemiluminescent reagents (ThermoFisher Scientific) using the Chemidoc MP with Image Lab software.

Statistics

Mean data given ±SD unless otherwise stated. Student's unpaired t-test used for comparison of two samples where data were normally distributed (Fig.s 3 & 6) and Wilcoxin-Mann-Whitney (WMW) test used where data were not normally distributed (Fig. 4). Linear dependence between two variables examined by Pearson's correlation coefficient analysis (e.g. Fig. 5). Significance level set at $P < 0.05$.

Results

Segments of single muscle fibres from the *vastus lateralis* muscle of Old and Young subjects were subjected to repeated load-release cycles in which the SR was depleted of all releasable Ca^{2+} by exposure to the 'full release solution' (containing 30 mM caffeine with low (0.05 mM) cytoplasmic Mg^{2+}) and then reloaded with Ca^{2+} by exposure for a set time (10 to 180 s) to a load solution at pCa 6.7 before again emptying the SR, as shown in Figure 1. The relative amount of Ca^{2+} accumulated by the SR could be gauged from the time-integral of the force response elicited in the full release solution, as described in detail previously (Lamboley *et al.*, 2013). In each skinned fibre, successive load-release cycles were carried out using load solutions containing 1, 3 and 10 mM free Mg^{2+}. When the Ca^{2+} loading of the SR was carried out in the presence of 1 mM free Mg^{2+}, close to the normal physiological level (Westerblad & Allen, 1992), the fibres from the Young subjects showed SR Ca^{2+} loading characteristics very similar to those reported previously for a similar cohort of young subjects (Lamboley *et al.*, 2013), with the half-time for maximal loading being approximately 22 s and 27 s in type I and type II fibres respectively (Fig. 2 B & Table 1). The relative amount of endogenous Ca^{2+} initially present in the SR, indicated by the force response upon first emptying the SR (e.g. Fig. 1), was ~52% and 40% of the maximal SR load level in those fibre types respectively (Fig. 2 B & Table 1), which also was very similar to that found previously in young subjects (Lamboley *et al.*, 2013).

Whilst the characteristics of SR Ca^{2+} accumulation in fibres of the Old subjects were in general similar to those in Young subjects, one key difference was that Ca^{2+} loading in the type I fibres in the presence of 1 mM Mg^{2+} started to show saturation at relatively earlier loading times (e.g. compare continuous line curves in Figures 2A & 2B). This could be seen both from the decrease in the half loading time (mean ± SD: 18.7 ± 4.7 s versus 21.9 ± 7.9 s in Old and Young type fibres, respectively) and the increase in the percent filling of the SR with 60 s loading (85.0 ± 6.1 % versus 81.3 ± 6.1% respectively of that reached with 180 s loading) (Table 1). Furthermore, it was apparent that the total amount of Ca^{2+} accumulated by the SR after 180 s loading was significantly lower in the type I fibres of Old subjects than in the type I fibres of Young subjects, as shown by the smaller size of the time integral of the force response elicited when releasing all the SR Ca^{2+} (Figure 3). [Note that in this assay the time integral of force response in each fibre was normalized to the maximal Ca^{2+}-activated force measured in that fibre (see Fig. 1), thereby taking into account the small difference in maximum force generation in the fibres of Young and Old subjects (Lamboley *et al.*, 2015)]. This latter finding is in accord with our previous absolute measurements of SR Ca^{2+} content showing that the amount of releasable Ca^{2+} in the SR after maximal loading was

significantly lower in type I fibres of Old subjects than in type I fibres of Young subjects (1.24 ± 0.12 versus 1.36 ± 0.15 mmol Ca^{2+} per litre fibre volume, respectively) (Lamboley *et al.*, 2015).

In contrast to the type I fibres, in type II fibres there was no difference in either the half loading time or the percentage SR filling at 60 s between Old and Young subjects (Table 1). Although the time integral of the force response elicited upon releasing all SR Ca^{2+} in the type II fibres was smaller in the Old subjects (Figure 3), it was possible that this was due at least in part to the lower Ca^{2+}-sensitivity of the contractile apparatus in type II fibres in Old subjects relative to that in Young subjects (Lamboley *et al.*, 2015).

Effect of raised cytosolic [Mg^{2+}]

The relatively rapid saturation of the SR Ca^{2+} loading curve in the presence of 1 mM Mg^{2+}, and the lower total SR Ca^{2+} accumulation, observed in the type I fibres of the Old subjects might both be explained by increased leakage of Ca^{2+} out of the SR occurring in parallel with the uptake. To test whether there was increased Ca^{2+} leakage through the RyRs, we examined the effects of raising cytoplasmic [Mg^{2+}] on the ability of the SR to accumulate Ca^{2+}. Raising the free [Mg^{2+}] from 1 mM to 3 or 10 mM slowed the rate of SR Ca^{2+} accumulation in fibres of both Young and Old subjects (e.g. Fig. 2), as expected from the known competitive effects of Mg^{2+} on Ca^{2+} pumping by SERCA (Chiesi & Inesi, 1981; Kabbara & Stephenson, 1994), but the fibres were still able to accumulate substantial amounts of Ca^{2+} provided that the loading time was sufficiently long. In the type I fibres of Young subjects the amount of Ca^{2+} accumulated by the SR after 180 s loading in the presence of 10 mM Mg^{2+} reached on average ~87% of that accumulated in 1 mM Mg^{2+} (e.g. Fig. 2); the individual values found in 15 type I fibres from 7 Young subjects are shown in Figure 4. In comparison, the type I fibres from Old subjects in the presence of 10 mM Mg^{2+} accumulated on average ~96% of that accumulated in 1 mM Mg^{2+}, and strikingly a number of the fibres actually accumulated more total Ca^{2+} in the SR when loaded in 10 mM Mg^{2+} than in 1 mM Mg^{2+} (e.g. Fig. 2A, and Fig. 4 showing 28 type I fibres from 7 Old subjects). The values for SR Ca^{2+} accumulation in 10 mM Mg^{2+} relative to 1 mM Mg^{2+} in the type I fibres of the Old subjects were significantly greater than in the Young subjects ($z = 2.91$, $P < 0.002$, Wilcoxin-Mann-Whitney test; non-parametric test used because values in Old subjects were not normally distributed). Furthermore, analyzing the data in terms of individual subjects (i.e. averaging the values found across all fibres in each given subject), also confirmed that the values for the type I fibres in the Old subjects were significantly greater than in the Young subjects ($P = 0.013$, 7 Old versus 7 Young subjects, Wilcoxin-Mann-Whitney test). It was also apparent that the type I fibres of the Old subjects with fastest saturation of Ca^{2+} loading in 1 mM Mg^{2+} (i.e. shortest time for half maximal

loading) displayed the greatest relative increase in Ca^{2+} accumulation in 10 mM Mg^{2+} (Figure 5), consistent with the saturating behaviour of the Ca^{2+} loading being due to increased SR Ca^{2+} leakage through the RyRs. Together, these data show that raising the cytosolic [Mg^{2+}] increased net Ca^{2+} accumulation by the SR to a greater degree in type I fibres of Old subjects than in type I fibres of Young subjects. In contrast, in type II fibres the effect of raising cytosolic [Mg^{2+}] on SR Ca^{2+} accumulation was no different between Old and Young subjects (Figure 4).

Effect of DTT treatment

We also examined whether SR Ca^{2+} accumulation was affected by treating fibres with the strong reducing agent, DTT (10 mM for 5 min). In each fibre the amount of Ca^{2+} accumulated by the SR with 180 s loading in the presence of 1 mM Mg^{2+} was measured both before and after the DTT treatment. DTT treatment resulted in a significant increase in SR Ca^{2+} accumulation in the type I fibres of Old subjects but not in any of the other cases (Fig. 6). Control experiments showed that, in the absence of DTT treatment, total SR Ca^{2+} accumulation normally declined slightly on successive load-release cycle (percentage accumulation on second control trial relative to first: 98 ± 2% in Young type I (n=7); 97 ± 5% Old type I (n=23); 94 ± 6% in Young type II (n=9); 99 ± 2% in Old type II fibres).

Densities of DHPRs and RyR1s

We used western blotting to examine the densities of the DHPRs (α1 subunit) and RyR1s in muscle of the Old and Young subjects. Unfractionated muscle homogenates from 11 Old and 10 Young subjects were run on each of two gels, together with mixed muscle samples for signal calibration (e.g. Figure 7). The relative densities of both the DHPRs and the RyR1s were very similar in the muscle homogenates from the Young and Old subjects, irrespective of whether the signals were normalized to actin or myosin (Table 2).

We also examined the DHPR and RyR1 densities in individual muscle fibres (e.g. Fig. 8), using a subset of the skinned fibre segments examined in the Ca^{2+} accumulation experiments. In the fibres of the four Young subjects examined, the densities of the DHPRs and the RyR1s were on average both ~50% higher in the type II fibres (n = 19) than in the type I fibres (n = 17). In fibres from the three Old subjects examined (29 type I and 12 type II) there was more disparity in the relative densities of the DHPRs and RyRs than in the fibres from the Young subjects, with a small proportion of the fibres displaying relatively high DHPR and low RyR density. Overall, the ratio of DHPRs to RyRs in the fibres of the Old subjects was, if anything, higher than that in the Young fibre sample.

Finally, the SERCA1 and SERCA2 densities in the muscle homogenates from the Old subjects were on average 61 ±19 % and 114 ±33 %, respectively, of that found in the muscle of the Young subjects; these values primarily reflected the decreased proportion of type II fibres and increased proportion of type I fibres in the muscle of the Old subjects (Lamboley *et al.*, 2015). The relative density of SERCA2 found in 6 type I fibres from four Old subjects was on average 94 ±24% of that found in 5 type I fibres from three Young subjects run on same gel. These data indicate that the SERCA density in individual muscle fibres was broadly similar in Young and Old subjects.

Discussion

This study examined the ability of the SR to accumulate Ca^{2+} in muscle fibres from active Old and Young adults, using mechanically skinned fibres in which the intracellular conditions could be set at a constant physiological level or varied similarly across all fibres. It was found that SR Ca^{2+} accumulation in the presence of 1 mM cytoplasmic Mg^{2+} saturated more rapidly (Fig. 2 & Table 1) and at a lower final level (Fig. 3) in type I fibres of Old subjects compared to similar fibres of Young subjects, indicative of increased SR Ca^{2+} leakage in the former. Importantly, raising the cytoplasmic [Mg^{2+}] from 1 to 10 mM, to specifically decrease Ca^{2+} leakage through the RyRs (Meissner *et al.*, 1986; Lamb *et al.*, 2001; Laver *et al.*, 2004), increased net SR Ca^{2+} accumulation to a significantly greater degree in the type I fibres of Old subjects than in the type I fibres of the Young subjects (Fig. 4), with some type I fibres from the Old subjects actually accumulating more total SR Ca^{2+} in 180 s loading in 10 mM Mg^{2+} than in 1 mM Mg^{2+} despite the strong competitive effects of the increased [Mg^{2+}] on SERCA Ca^{2+} pumping (Chiesi & Inesi, 1981; Kabbara & Stephenson, 1994). Furthermore, treatment with the reducing agent DTT also increased net Ca^{2+} accumulation in the type I fibres of Old subjects and not in the other groups (Fig. 6), with the magnitude of the increase being similar to that observed with raised [Mg^{2+}] (Fig. 4). These data demonstrate i) that there is increased Ca^{2+} leakage out of the SR in the type I fibres of Old human subjects relative to that in type I fibres of Young subjects, ii) that the leakage occurs primarily through the RyRs, and iii) that strong reducing treatment inhibits this RyR Ca^{2+} leakage, increasing net SR Ca^{2+} accumulation. These findings are analogous to those made in muscle of aged mice (in muscles with predominantly type II fibres), where there was increased oxidation/nitrosylation of the RyRs, leading to greater SR Ca^{2+} leak and decreased SR Ca^{2+} content, which could be reversed by in-vitro treatment with DTT (see Introduction) (Andersson *et al.*, 2011; Umanskaya *et al.*, 2014).

The present study found no evidence of increased Ca^{2+} leakage in the type II fibres of the Old subjects; specifically, there was no significant difference from the type II fibres of Young subjects in regard to the time course of Ca^{2+} accumulation (Table 1) or the effect of raising [Mg^{2+}] to 10 mM (Fig. 4) or the effect of DTT treatment (Fig. 6). Nevertheless, we have previously observed that the maximal level of SR Ca^{2+} loading is lower in type II fibres of Old compared to Young subjects (Lamboley *et al.*, 2015). One possible explanation for this apparent discrepancy is that the Ca^{2+} accumulation assay used in the present experiments may be a relatively insensitive measure of SR Ca^{2+} leakage, because the [Ca^{2+}] within the skinned fibres during the loading was buffered with only a moderate [EGTA] (1 mM total at pCa 6.7) and hence Ca^{2+} leaking out of the SR would have increased the local [Ca^{2+}] near the SR, in turn leading to increased uptake by the SERCA and recovery of some of leaked Ca^{2+}. Consequently, it is possible that SR Ca^{2+} leakage was increased

also in the type II fibres in the Old subjects but that this local Ca^{2+} flux in the type II fibres was not detected with the whole fibre Ca^{2+} accumulation assay used here, possibly in part because the SERCA density in the type II fibres (almost exclusively SERCA1a (Lamboley *et al.*, 2014)) is likely substantially higher than in type I fibres (almost exclusively SERCA2a), assuming that human fibres show similar relative SERCA density distribution as seen in rat and rabbit muscle (Leberer & Pette, 1986; Wu & Lytton, 1993). The level of Ca^{2+} buffering used in the assay here was intentionally kept relatively low in order to study SR Ca^{2+} leakage at close to normal resting intracellular [Ca^{2+}] without grossly perturbing local Ca^{2+} movements by dampening Ca^{2+}-activation or Ca^{2+}-inactivation of the RyRs.

Irrespective of whether there was some level of undetected SR Ca^{2+} leakage in the type II fibres, the present study clearly demonstrates that in the type I fibres of the Old subjects there is substantially increased Ca^{2+} leakage through the RyRs that results from reversible oxidative modification of cysteine residues on the RyRs because it can be prevented by treatment with DTT. It is noteworthy that our preceding study on the contractile apparatus properties in muscle of Old and Young subjects (Lamboley *et al.*, 2015) found that maximum specific force, Ca^{2+} sensitivity and the effects of S-glutathionylation on Ca^{2+}-sensitivity were all decreased in the type II fibres in the Old subjects, but not in the type I fibres, and that these particular effects in the type II fibres likely all reflected irreversible oxidative damage of the contractile apparatus. It was noted that type I fibres were likely to be less susceptible to such irreversible oxidative damage than type II fibres because type I fibres contain higher levels of GSH and superoxide dismutase (at least in rats) (Ji *et al.*, 1992; Masuda *et al*., 2003). This may well also account for the present findings. The higher GSH level in type I fibres would be expected to decrease the deleterious actions of superoxide, hydroxyl and peroxynitrite, all of which can potentially cause irreversible oxidation of cysteine (e.g. sulphonation) and other residues. However, the reaction of reactive oxygen or nitrogen species (ROS/RNS) with the GSH would be expected to result via various pathways in increased S-glutathionylation of cysteine residues on the contractile apparatus (Klatt & Lamas, 2000; Lamb & Westerblad, 2011; Mollica *et al.*, 2012), thereby protecting them from irreversible oxidative modification, but also to result in increased S-glutathionylation of the RyRs, which has been shown to decrease the inhibitory effect of Mg^{2+} on the RyRs (Aracena *et al.*, 2003), which would increase resting Ca^{2+} leakage. Furthermore, there would likely also be increased generation of GSNO, which has been shown to lead to S-nitrosylation of the RyRs, increasing their sensitivity to Ca^{2+} activation (Aracena *et al.*, 2003), which would also increase Ca^{2+} leakage. Similar reversible effects also occur with mild oxidation of the RyRs by hydrogen peroxide (by sulphenation or cross-linking of cysteine residues) (Favero *et al.*, 1995; Posterino *et al.*, 2003; Aracena-Parks *et al.*,

2006). In summary, the increase in Ca^{2+} leakage through the RyRs may be due to the single or combined effect of a variety of different types of reversible oxidative modifications of the RyRs. In view of this complexity, and the present lack of definitive information about which and how many of the many different cysteine residues on the RyR are involved in each of these types of oxidative modifications (Aracena-Parks *et al.*, 2006), we did not attempt to identify specific oxidative modifications of the RyRs, particularly given that the increased Ca^{2+} leakage could be due to modification of only a small proportion of the total pool of RyRs. Finally, we note that the importance of the specific cellular anti-oxidant levels in influencing whether ROS/RNS affect contractile function or SR Ca^{2+} release is vividly shown by studies on single muscle fibres in which it was found that increased expression of superoxide dismutase or application of various different combinations of ROS/RNS-neutralizing compounds differentially altered the relative susceptibility of the contractile apparatus and the Ca^{2+} release process to oxidative modification (Bruton *et al.*, 2008; Cheng *et al.*, 2015).

DHPR and RyR densities

We also examined the densities of both the DHPR α1 subunits and the RyRs in the muscle of the Old relative to the Young subjects, because it has been reported that there is a loss of DHPRs with age in rodent and rabbit muscle (Renganathan *et al.*, 1997; Ryan *et al.*, 2000). It has been suggested that such loss of DHPRs underlies the decreased SR Ca^{2+} release seen in both rodent (Wang *et al.*, 2000) and human muscle (Delbono *et al.*, 1995), and it might also increase SR Ca^{2+} leakage because of a decrease in the resting inhibition exerted by the DHPRs on the RyRs (Zhou *et al.*, 2006; Eltit *et al.*, 2011; Robin & Allard, 2012). However, in the human tissue here we found no evidence of a differential loss of DHPRs or indeed of any overall loss of either DHPRs or RyRs in the muscle of the Old subjects; on average the density of the DHPRs and RyRs in total muscle homogenates from the 11 Old subjects was ~0.98 and ~0.96 times, respectively, of that found in the muscle of the 10 Young subjects (Table 2). These values are similar to the 'expected' value of ~0.94 derived taking into account the increase in the proportion of type I fibres in the Old subjects (ratio of type I to type II changing from ~50:50 to ~65:35 (Lamboley *et al.*, 2015)) and the fact that the density of DHPRs and RyRs are both ~50% lower in type I fibres compared to type II fibres. A previous study in SR vesicles also concluded that the DHPR density was unchanged in muscle of aged human subjects (Ryan *et al.*, 2003). Furthermore, in the relatively small set of single fibres examined here from a subset of the subjects, the DHPR density was if anything somewhat higher in the fibres of the Old subjects.

Conclusion

The findings in this study demonstrate that there is increased leakage of Ca^{2+} out of the SR through the RyRs in type I muscle fibres in aged humans, due to reversible oxidative modification of the RyRs, and that this Ca^{2+} leakage is probably the primary cause of the decreased SR Ca^{2+} content seen in such fibres (Lamboley *et al.*, 2015). Such SR Ca^{2+} leakage and depletion in human fibres is analogous to that observed in muscle fibres of aged mice (Andersson *et al.*, 2011; Umanskaya *et al.*, 2014), and possibly likewise may arise from a self-reinforcing in which Ca^{2+} leakage through the RyRs leads to increased ROS production by the mitochondria, which in turn further exacerbates the RyR leakage. The increase in local [Ca^{2+}] within the triad junction may well also contribute to the decreased SR Ca^{2+} release observed in muscle of aged humans and rodents (Delbono *et al.*, 1995; Wang *et al.*, 2000) by increasing the activity of Ca^{2+}-dependent proteases (calpains), leading to disruption of the normal coupling between the DHPRs and the RyRs (Lamb *et al.*, 1995; Murphy *et al.*, 2013). This, together with the direct effects of the SR Ca^{2+} depletion on Ca^{2+} release (Posterino & Lamb, 2003), could be expected to have significant deleterious effects on the muscle performance in aged individuals in addition to those arising from damage or modification of contractile apparatus properties (see Lamboley *et al*. (2015) and references therein).

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Competing interests

The authors declare that they have no competing interests.

Author contributions

Muscle biopsies were performed at Victoria University, and biochemical and physiological measurements on skinned fibres made at La Trobe University. VLW, CRL and MJM were responsible for selection, care and testing of human subjects, and obtaining muscle biopsies from the subjects. Skinned fibre experiments were designed and analysed by GDL and CRL and carried out by CRL, and RMM and GDL were responsible for Western blotting procedures and analysis with assistance of VLW. CRL and GDL 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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Table 1. SR Ca^{2+} loading properties of skinned muscle fibres from Young and Old subjects. Mean (±SD) of endogenous SR Ca^{2+} load level (Ca_{endo}) in mechanically skinned muscle fibres from Young and Old subjects, and half time for SR Ca^{2+} loading in presence of 1 mM cytoplasmic Mg^{2+}, derived as in Figures 1 & 2. 'n' denotes number of fibres and 'N' the number of subjects. '#' indicates that value in Old group is significantly different from matching value in Young group, and '*' indicates value for type II fibres is significantly different from that in type I fibres in same age group (Student's two tailed t-tests). Note that 60 s load time measurements were not made in some of the fibres for which loading curves were determined.

	Type I fibres		Type II fibres	
	Young	Old	Young	Old
Ca_{endo} (% max)	52 ± 9 (n=25; N=10)	55 ± 9 (n=28; N=7)	40 ± 7 * (n=30; N=10)	40 ± 9 * (n=14; N=8)
Half time (s)	21.9 ± 7.9 (n=34; N=12)	18.7 ± 4.7 # (n=39; N= 12)	26.8 ± 10.6 * (n=41; N=13)	25.2 ± 6.6 * (n=14; N= 8)
% full at 60 s load	81.3 ± 6.1 (n=22; N= 9)	85.0 ± 6.1 # (n=31; N= 7)	77.5 ± 11.7 * (n=20; N= 7)	78.8 ± 7.7 * (n=10; N= 6)

Table 2. DHPR and RyR densities in muscle homogenates of Young and Old subjects. Mean ± SD of relative density of DHPRs and RyR1s, obtained by western blotting as in Figure 7. Values for each subject normalized to average of that found in the samples of all Young subjects on the same gel. Values represent average of duplicate measurements from different gels.

	Old (n=11)	**Young (n=10)**
DHPR density		
normalized to myosin	0.98 ± 0.26	1.00 ± 0.22
normalized to actin	0.97 ± 0.31	1.00 ± 0.31
RyR1 density		
normalized to myosin	0.94 ± 0.26	1.00 ± 0.25
normalized to actin	0.97 ± 0.32	1.00 ± 0.32

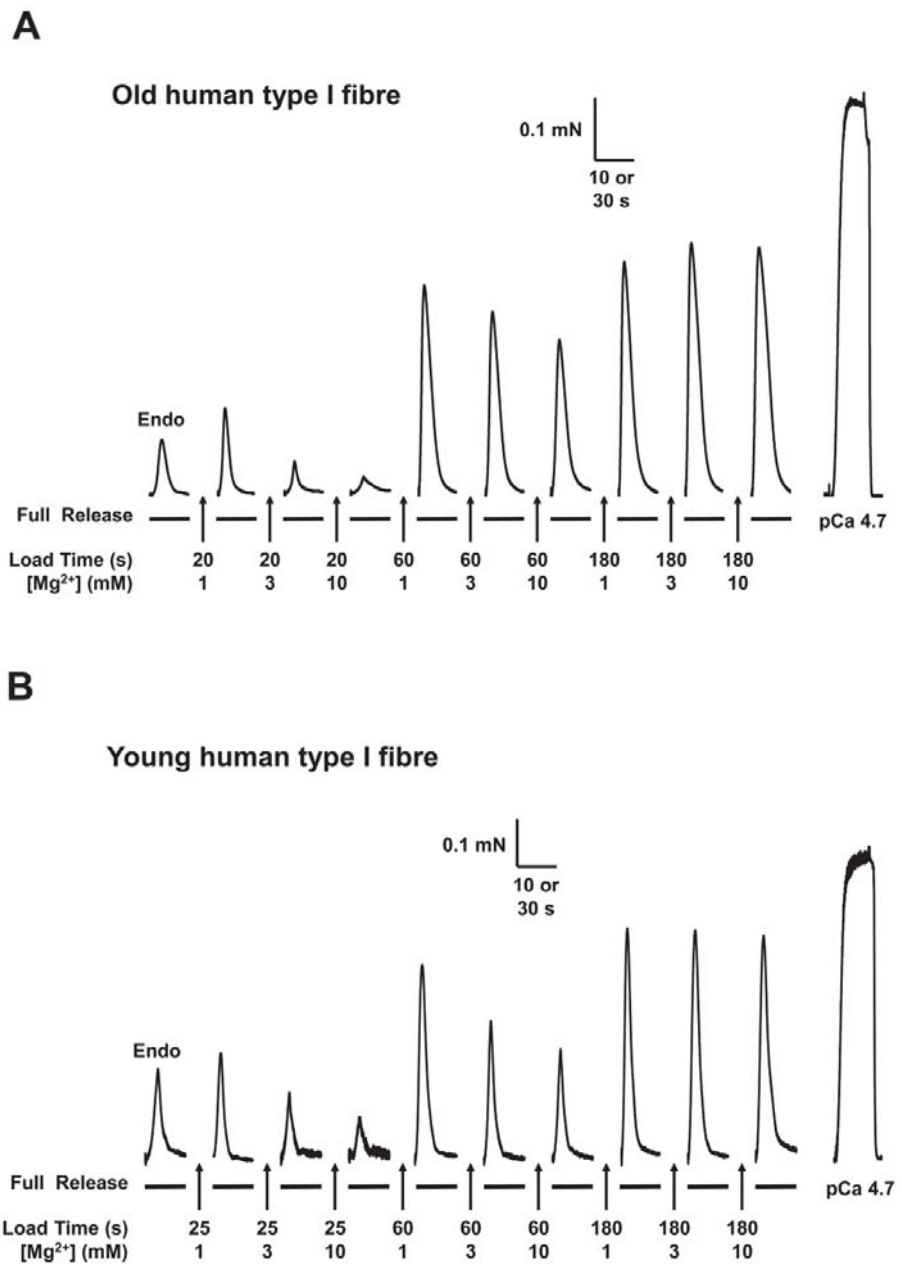

Figure 1. Effect of cytoplasmic [Mg^{2+}] on SR Ca^{2+} accumulation in a type I muscle fibre from an Old and a Young subject.

Force responses in type I fibres from Old (A) and Young (B) subject. In each case the skinned fibre was subjected to repeated load-release cycles in which the SR was loaded with Ca^{2+} for the indicated time in a load solution at pCa 6.7 with 1, 3 or 10 mM cytoplasmic free Mg^{2+} and then the SR Ca^{2+} released by exposing the fibre to 'full release solution' (see Methods). The first response in each sequence was that elicited upon releasing the endogenous SR Ca^{2+} ('Endo'). The final response in each sequence shows maximum force elicited by directly activating the contractile apparatus in a heavily Ca^{2+}-buffered solution at pCa 4.7 (see Methods). Time scale: 10 s during SR Ca^{2+} release, and 30 s for maximum Ca^{2+}-activated force.

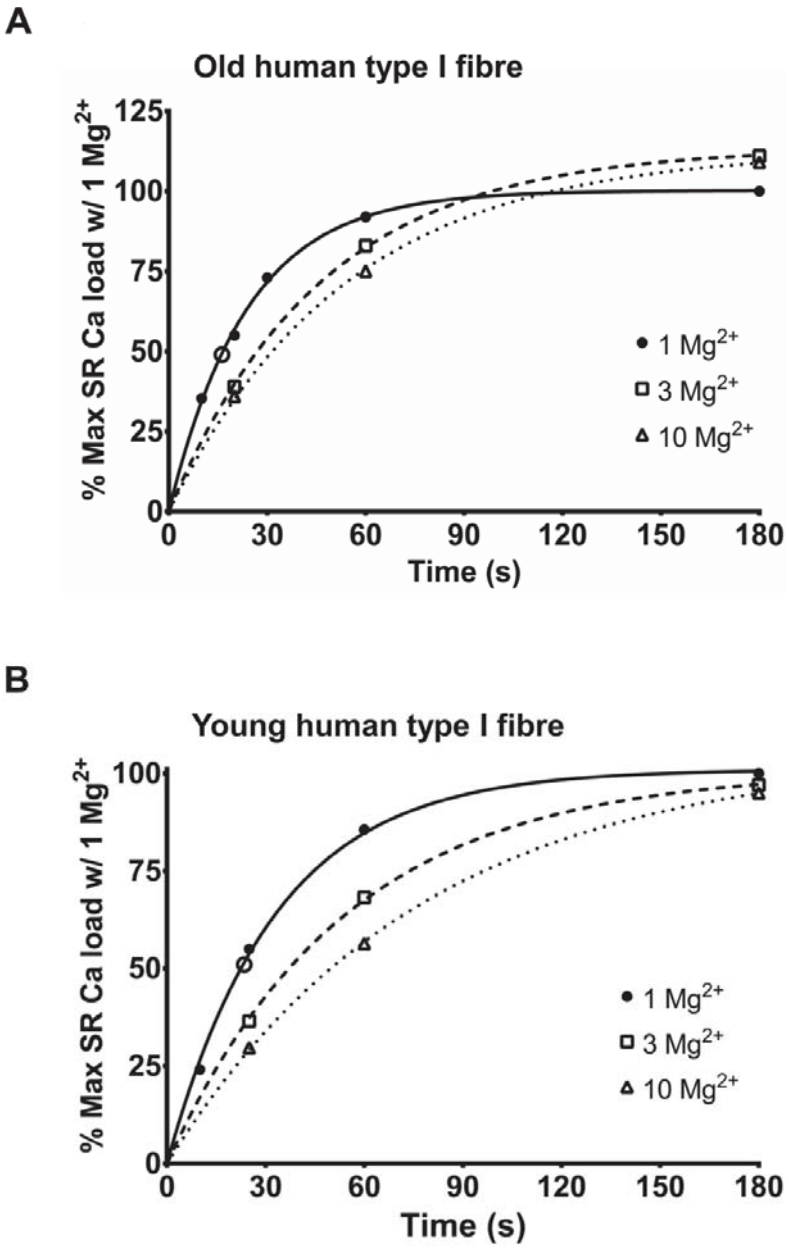

Figure 2. SR Ca^{2+} loading characteristics in type I fibre from an Old and a Young subject. Plots of relative SR Ca^{2+} content versus load time when Ca^{2+} loading a type I fibre from an Old (A) or Young (B) subject in presence of 1, 3 or 10 mM Mg^{2+}, derived from records in Figures 1A and 1B, respectively. Each value expressed as percentage of maximal SR Ca^{2+} capacity found with 180 s loading in 1 mM Mg^{2+}. Data sets fitted with best-fit single exponential function. Data have been adjusted for the negative ordinate intercept resulting from releasing SR Ca^{2+} in the presence of 0.5 mM EGTA (see Methods). Half-time for Ca^{2+} loading with 1 mM Mg^{2+} present was ~16 s and ~23 s in A and B, respectively. Open circle symbols on fitted functions for 1 mM Mg^{2+} indicate relative force–time integral found upon releasing the endogenous Ca^{2+} content, and correspond to ~49% and 51% of maximum content in fibres in A and B, respectively.

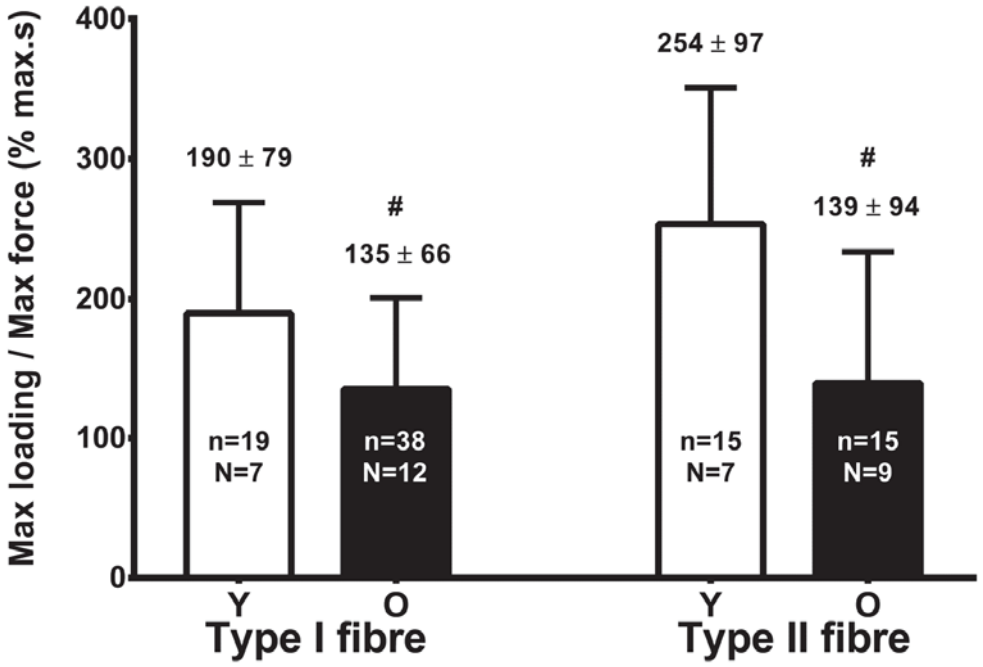

Figure 3. Measure of maximum SR Ca^{2+} loading in 1 mM Mg^{2+} in fibres from Young (Y) and Old (O) subjects.

Mean (+SD) of time integral of force response upon full release of SR Ca^{2+} after loading fibres for 180 s in presence of 1 mM Mg^{2+} (see Figure 1); values normalised to maximum Ca^{2+}-activated force measured in same fibre. 'n' denotes number of fibres and 'N' the number of subjects. '#' indicates that value in Old group is significantly different from matching value in Young group (Student's paired two tailed t-test).

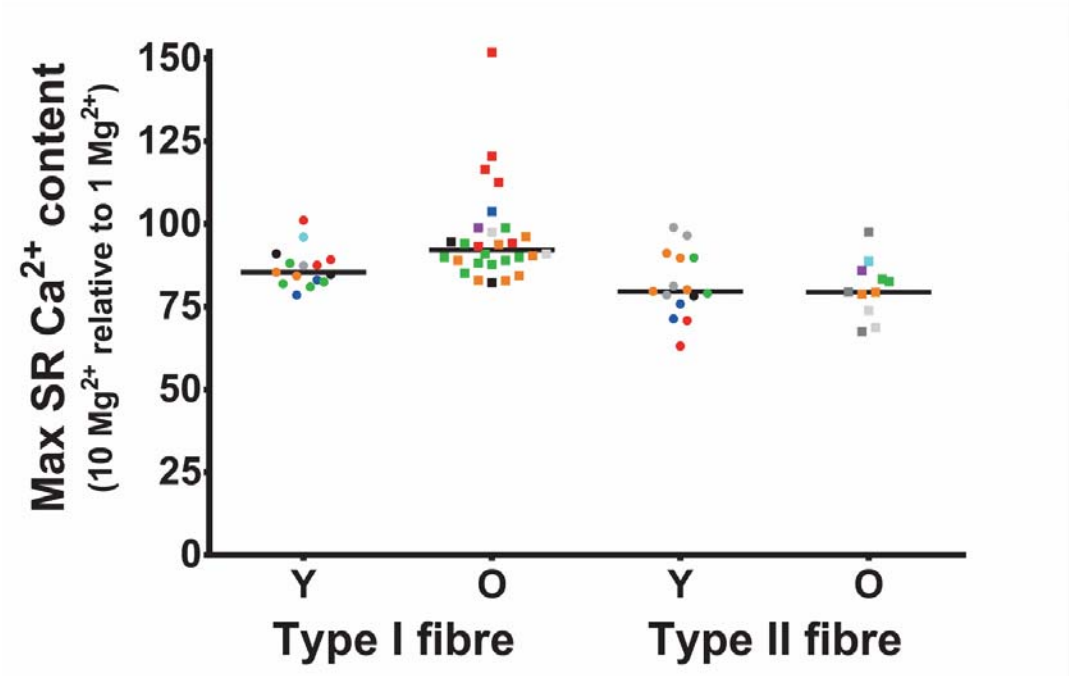

Figure 4. Effect of raised [Mg^{2+}] on maximal SR Ca^{2+} accumulation in individual fibres. Values denote SR Ca^{2+} content accumulated after 180 s loading in presence of 10 mM cytoplasmic Mg^{2+}, expressed as a percentage of that accumulated in the same fibre in 1 mM Mg^{2+}, measured as in Figures 1 & 2. Each particular symbol labels fibres obtained from a given subject (9 Old (O) and 9 Young (Y) subjects). Horizontal lines indicate median value in each case. The presence of 10 mM Mg^{2+} resulted in greater SR Ca^{2+} accumulation in type I fibres of Old subjects compared to that in type I fibres of Young subjects (see text).

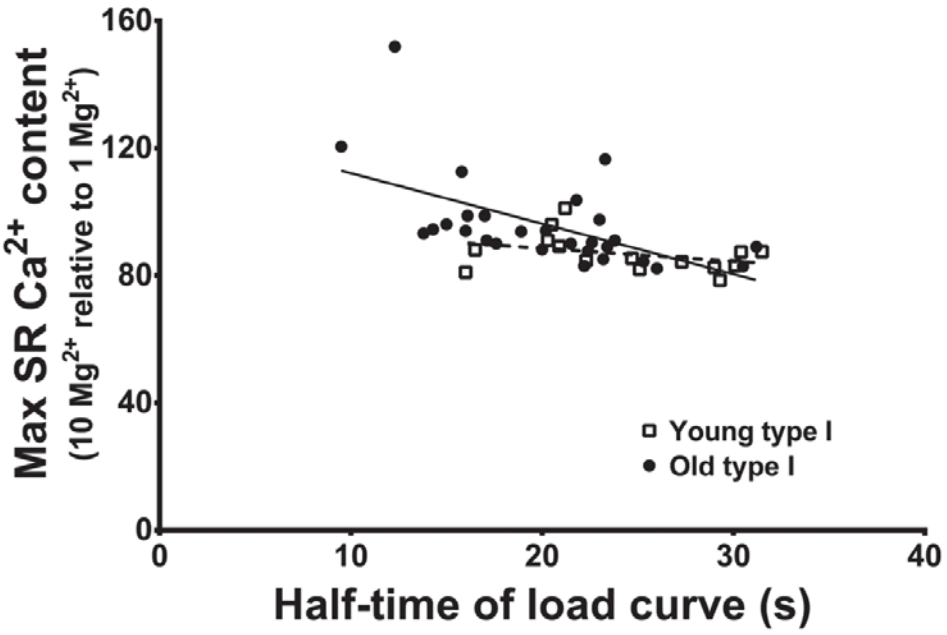

Figure 5. Inverse relationship between SR Ca^{2+} leak measure and half-time for SR Ca^{2+} accumulation in type I fibres of Old subjects.
Relative SR Ca^{2+} accumulation in presence of 10 mM cytoplasmic Mg^{2+} (expressed as a percentage of that accumulated in 1 mM Mg^{2+}) in individual type I fibres from Young and Old subjects versus half-time of SR Ca^{2+} loading in presence of 1 mM Mg^{2+} (measured as in Figure 2). Pearson's correlation coefficient (r) was -0.56 for the fibres from the Old subjects ($P < 0.05$) indicating a negative correlation, and -0.35 for the Young type I fibres ($P > 0.05$, no significant correlation). Line of best fit shown for each case.

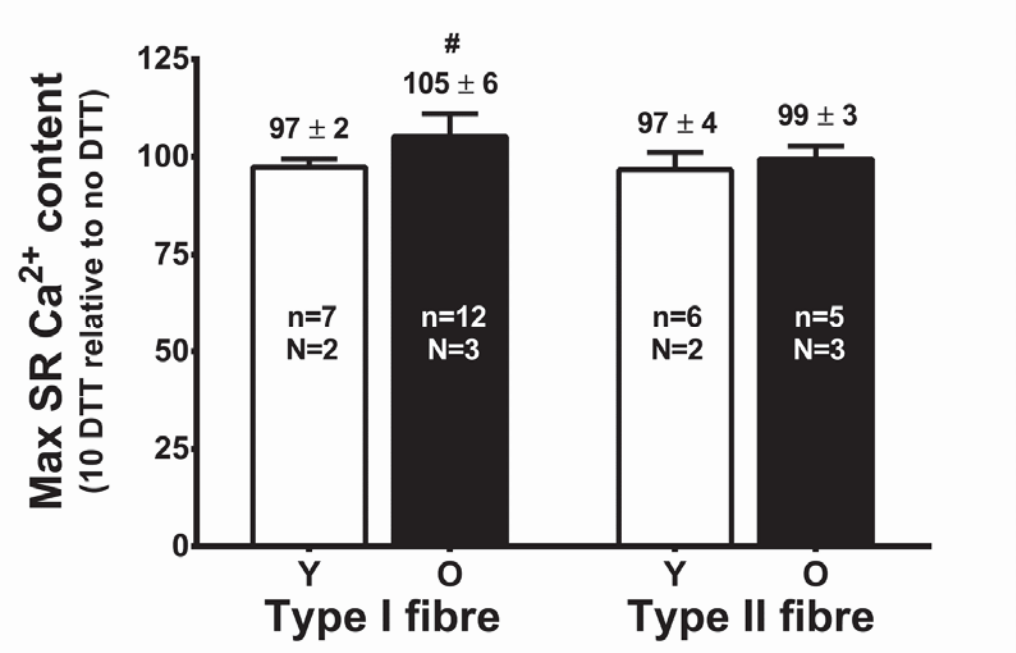

Figure 6. Effect of reducing treatment (10 mM DTT) on SR Ca^{2+} accumulation.
Mean (+SD) of maximum SR Ca^{2+} accumulation (180 s load time) in 1 mM Mg^{2+} in type I and type II fibres from Young (Y) and Old (O) subjects following DTT treatment (10 mM for 5 min), expressed as a percentage of that accumulated in the same fibre before DTT treatment. 'n' denotes number of fibres and 'N' the number of subjects. '#' indicates value is significantly greater than 100% ($P < 0.025$, Student's one-tailed t-test). Note that a small decrease in the amount of Ca^{2+} accumulation is observed upon successive measurements even without DTT treatment (see text).

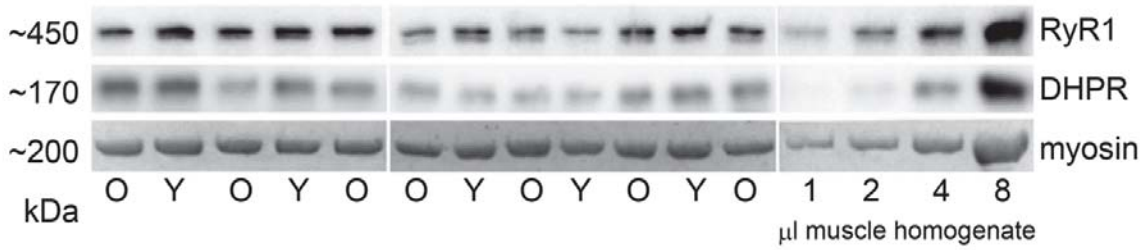

Figure 7. DHPR and RyR density in muscle homogenates from Old and Young subjects. Upper two panel sets: western blots for DHPR α1 subunit and RyR1 in muscle homogenates from 7 Old (O) and 5 Young (Y) subjects. Right hand lanes contained 1 to 8 μl of a mixed homogenate of muscle from all subjects, used for signal calibration. Bottom panel: myosin band on coomassie-stained post-transfer gel. 4-12% BIS/TRIS Criterion precast gel.

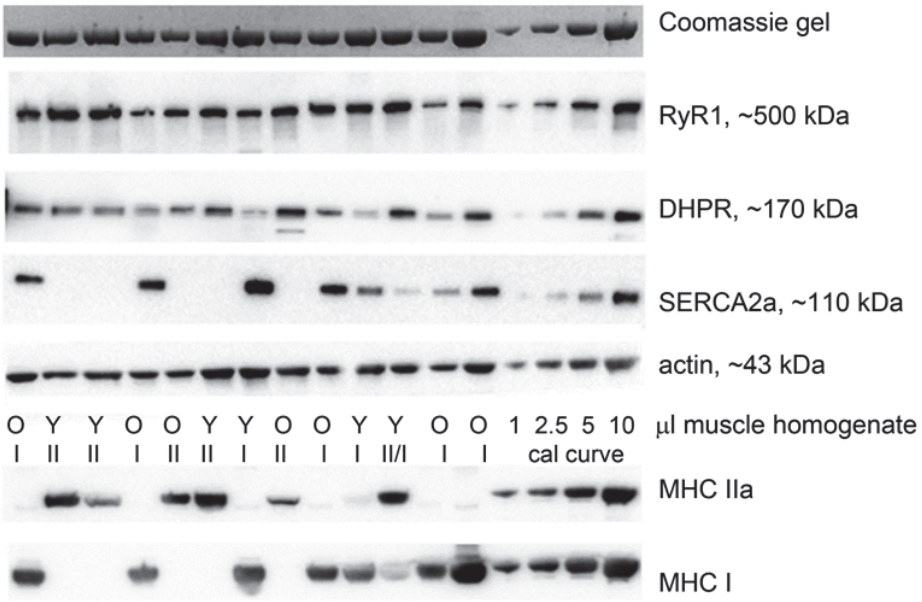

Figure 8. DHPR and RyR density in single muscle fibres from Old and Young subjects. Western blots for DHPRα1, RyR1, SERCA2a, actin, MHCI and MHCIIa in individual fibres from Young (Y) and Old (O) subjects (single skinned fibre segment in each lane), with four right-hand lanes containing 1 to 10 µl of a mixed homogenate of muscle from all subjects, used for signal calibration. Labels I and II denotes fibres containing predominantly MHCI and MHCIIa respectively; label II/I denotes a mixed fibre containing substantial amounts of both MHCIIa and MHCI. 4-12% Bis/Tris Criterion precast gel. Top panel shows myosin band on coomassie-stained post-transfer gel.