

Ca²⁺ leakage out of the sarcoplasmic reticulum is increased in type I skeletal muscle fibres in aged humans

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Key points

- The amount of Ca²⁺ stored in the sarcoplasmic reticulum (SR) of muscle fibres is decreased in aged individuals, and an important question is whether this results from increased Ca²⁺ leakage out through the Ca²⁺ release channels (ryanodine receptors; RyRs).
- The present study examined the effects of blocking the RyRs with Mg²⁺, or applying a strong reducing treatment, on net Ca²⁺ accumulation by the SR in skinned muscle fibres from Old (~70 years) and Young (~24 years) adults.
- Raising cytoplasmic [Mg²⁺] and reducing treatment increased net SR Ca²⁺ 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 Ca²⁺ leakage from the SR in muscle fibres in Old subjects, which probably deleteriously affects normal muscle function both directly and indirectly.

Abstract The present study examined whether the lower Ca²⁺ 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 Ca²⁺ out of the SR through the Ca²⁺ release channels/ryanodine receptors (RyRs) and due to oxidative modification of the RyRs. SR Ca²⁺ accumulation in mechanically skinned muscle fibres was examined in the presence of 1, 3 or 10 mM cytoplasmic Mg²⁺ because raising [Mg²⁺] strongly inhibits Ca²⁺ efflux through the RyRs. In type I fibres of Old subjects, SR Ca²⁺ accumulation in the presence of 1 mM Mg²⁺ approached saturation at shorter loading times than in Young subjects, consistent with Ca²⁺ leakage limiting net uptake, and raising [Mg²⁺] to 10 mM in such fibres increased maximal SR Ca²⁺ accumulation. No significant differences were seen in type II fibres. Treatment with dithiothreitol (10 mM for 5 min), a strong reducing agent, also increased maximal SR Ca²⁺ accumulation at 1 mM Mg²⁺ in type I fibres of Old subjects but not in other fibres. The densities of dihydropyridine receptors and RyRs were not significantly different in muscles of Old relative to Young subjects. These findings indicate that Ca²⁺ leakage from the SR is increased in type I fibres in Old subjects by reversible oxidative modification of the RyRs; this increased SR Ca²⁺ leak is expected to have both direct and indirect deleterious effects on Ca²⁺ movements and muscle function.

(Resubmitted 29 July 2015; accepted after revision 11 November 2015; first published online 18 November 2015)

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Abbreviations DHPR, dihydropyridine receptor; GSH, glutathione; HDTA, hexa-methylene-diamine-tetraacetate; MHC, myosin heavy chain; pCa, $-\log_{10} [\text{Ca}^{2+}]$; RNS, reactive nitrogen species; ROS, reactive oxygen species; RyR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum Ca²⁺-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 the result of a loss of total muscle mass, as well as changes within the muscle fibres themselves. The decline in single muscle fibre performance in old age may be the result of some level of dysfunction in one or more of the steps in the excitation–contraction coupling sequence (Bottinelli & Reggiani, 2000; Rebbeck *et al.* 2014), including disruption of the coupling between the dihydropyridine receptors (DHPRs) and the ryanodine receptor (RyR)/Ca²⁺ release channels (Delbono *et al.* 1995; Wang *et al.* 2000), reduced storage and release of sarcoplasmic reticulum (SR) Ca²⁺ (Jimenez-Moreno *et al.* 2008; Andersson *et al.* 2011; Lamboley *et al.* 2015) or a reduction in both the Ca²⁺-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 Ca²⁺ 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 Ca²⁺ efflux from the SR, resulting in increased Ca²⁺ 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 Ca²⁺ leakage through the RyRs causes a decreased level of Ca²⁺ 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).

In the present study, we examined whether there is increased Ca²⁺ leakage through the RyRs in the skeletal muscle fibres of aged humans, which could help account for the reduced SR Ca²⁺ content in such fibres (Lamboley *et al.* 2015). This was investigated by examining the SR Ca²⁺ 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 [Mg²⁺] to increase SR Ca²⁺ accumulation. Ca²⁺ efflux through the RyRs is strongly inhibited by cytoplasmic Mg²⁺ (Meissner *et al.* 1986; Laver *et al.* 2004). In the presence of 1 mM Mg²⁺, which represents the normal cytoplasmic concentration, the resting Ca²⁺ 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). Raising the [Mg²⁺] from 1 to 3 mM decreases Ca²⁺ efflux by a further 5-fold in heavily Ca²⁺ loaded SR vesicles (Meissner *et al.* 1986) and strongly inhibits the ability of caffeine and Ca²⁺ to induce Ca²⁺ release in skinned fibres and single RyRs (Lamb *et al.*

2001; Laver *et al.* 2004). We hypothesized that SR Ca²⁺ leakage is elevated in muscle fibres in Old subjects and would be decreased by raising the cytoplasmic [Mg²⁺] from 1 mM to 10 mM, thereby producing an appreciable net increase in Ca²⁺ accumulation in fibres of Old subjects but not Young subjects. We further hypothesized that the increased SR Ca²⁺ 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 because decreased DHPR density has been observed in muscle of aged mice (Renganathan *et al.* 1997) and was proposed to be the cause of the reduced SR Ca²⁺ release seen in both mouse (Wang *et al.* 2000) and human muscle fibres (Delbono *et al.* 1995). 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

The present study was approved by the Human Research Ethics Committees of Victoria University and La Trobe University, and conformed with the *Declaration of Helsinki*. The muscle tissue used was obtained as part of the study described in detail by Lamboley *et al.* (2015), supplemented with tissue from three additional Young subjects. A total of nineteen Young (12 males and seven females) and nineteen Old adult subjects (13 males and six females) provided their written informed consent. The mean ± SD age 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 as performed by an experienced medical practitioner. After injection of a local anaesthetic (1% lidocaine) into the skin and fascia, a small incision was made in the middle third of the vastus lateralis muscle of each subject and a muscle sample was 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 subsequent analysis. Physiological measurements were made on single fibres from a total of 13 Young (nine males and four females) and 14 Old subjects (10 males and four females), who were all fibre-typed by western blotting (see below). Western blotting of muscle homogenates was performed on tissue

from a total of 10 Young (four males and six females) and 11 Old subjects (seven males and four females), using the same homogenate tissue as that reported in Lamboley *et al.* (2015), with fibres from four of these Young subjects and six 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): 50 hexa-methylene-diamine-tetraacetate (HDTA²⁻) (Fluka, Buchs, Switzerland); 8 total ATP, 36 Na⁺, 126 K⁺, 8.5 total Mg²⁺ (giving 1 mM free [Mg²⁺]), 10 creatine phosphate, 0.05 total EGTA and 90 Hepes; pH 7.1 and pCa (-log₁₀ [Ca²⁺]) ~7.1, except where stated. Where required, the SR of the skinned fibre was totally depleted of all releasable Ca²⁺ by exposure to the 'full release solution', which was similar to the K-HDTA solution but with 30 mM caffeine, 0.05 mM free Mg²⁺ (total Mg²⁺ of 2.1 mM) and 0.5 mM free EGTA (pCa 8.5) present to chelate released Ca²⁺. The SR was reloaded with Ca²⁺ by exposing the fibre for a set time (10–180 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 Mg²⁺ or with 12 or 22.7 mM total Mg²⁺ to give 3 and 10 mM free Mg²⁺, respectively (Lamb & Stephenson, 1991). The maximum force was determined in a solution similar to HDTA solution but with 50 mM EGTA and 49.5 mM Ca²⁺ (pCa 4.7) and 8.1 mM total Mg²⁺ to maintain the free [Mg²⁺] at 1 mM.

Ca²⁺ uptake and release protocol

The examination of Ca²⁺ accumulation by the SR in the skinned fibres was similar to that described previously

(Lamboley *et al.* 2013). Because the muscle fibres were kept and skinned under paraffin oil, the SR initially retained its endogenous level of Ca²⁺. Each skinned fibre segment was first bathed for 2 min in the standard K-HDTA solution to wash out all diffusible Ca²⁺ buffers present endogenously in the cytoplasm. The SR was then depleted of all of its releasable Ca²⁺ by exposing the fibre to the full release solution (see above). The fibre was then washed for 1 min 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 [Mg²⁺] 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 Ca²⁺ 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 Ca²⁺ content initially present in the fibre, and the responses on subsequent load–release cycles indicated the SR content for the respective loading time (Fig. 1). The relationship between the time integral of the force response and loading 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 Ca²⁺ 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 loading time (Lamboley *et al.* 2013).

Western blotting

Whole muscle homogenates from Young (*n* = 10) and Old (*n* = 11) subjects comprised 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 1 x solubilizing buffer, which contained 0.125 M Tris-HCl, 10% glycerol, 4% SDS, 4 M urea, 1% mercaptoethanol and ~0.001% bromophenol blue (pH 6.8) diluted (2:1 v/v) with double distilled water, as described previously (Murphy *et al.* 2011; Lamboley *et al.* 2013). Fibres were stored at -80°C until analysed by western blotting. For both whole muscle homogenates or isolated individual fibres, a four- to five-point calibration curve consisting of the same mixed muscle homogenate was 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 200 V with a Mops running buffer (1 M Mops, 1 M Tris base, 69 mM SDS and 20.5 mM EDTA), and with 150 μ l of β -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 (100 V 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 (BioRad). Membranes were treated with Miser antibody

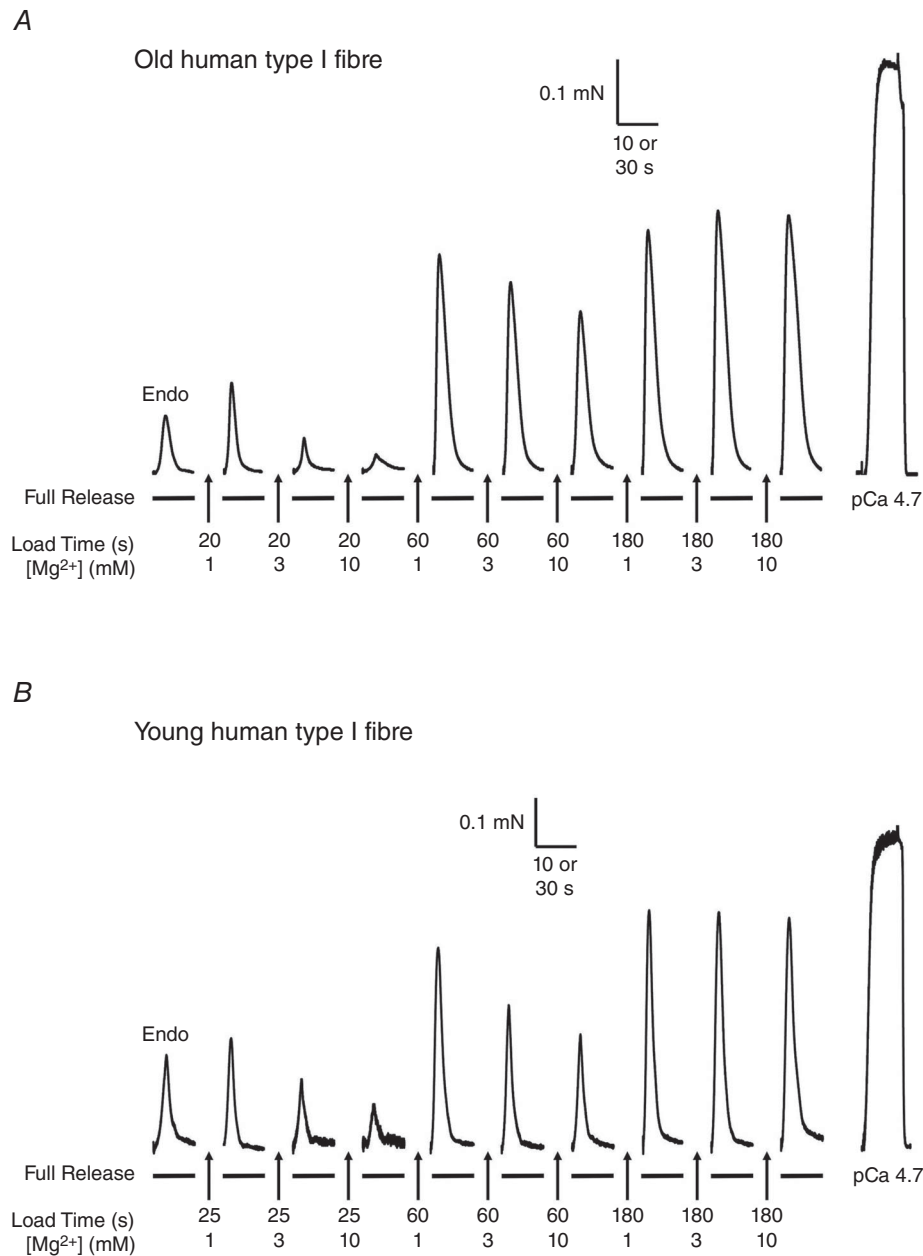


Figure 1. Effect of cytoplasmic [Mg²⁺] on SR Ca²⁺ accumulation in a type I muscle fibre from an Old and a Young subject

Force responses in type I fibres from Old (A) and Young (B) subjects. In each case, the skinned fibre was subjected to repeated load–release cycles in which the SR was loaded with Ca²⁺ for the indicated time in a load solution at pCa 6.7 with 1, 3 or 10 mM cytoplasmic free Mg²⁺ and then the SR Ca²⁺ 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 Ca²⁺ (‘Endo’). The final response in each sequence shows the maximum force elicited by directly activating the contractile apparatus in a heavily Ca²⁺-buffered solution at pCa 4.7 (see Methods). Time scale: 10 s during SR Ca²⁺ release; 30 s for maximum Ca²⁺-activated force.

extender solution (ThermoFisher Scientific, Scoresby, VIC, Australia), and then blocked in 5% skim milk in Tris-buffered saline with Tween for 1–2 h at room temperature. Membranes were exposed to primary antibodies overnight at 4°C and 2 h at room temperature. The antibodies used and their dilutions were: myosin heavy chain IIa [MHCIa, mouse IgG; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA: A4.74, dilution 1:400], myosin heavy chain I (MHCI, mouse IgM; DSHB: A4.840, dilution 1:100), ryanodine receptor 1 (RyR1, mouse; DSHB: 34C, dilution 1:100), dihydropyridine receptor (DHP, mouse; DSHB: IID5EI, dilution 1:400), SERCA1 (mouse; DSHB: CaF2-5D2,

dilution 1:1000) and SERCA2a (rabbit; Badrilla, Leeds, UK, dilution 1:5000). The HRP secondary antibodies used were goat anti-mouse HRP (ThermoFisher Scientific: PIE31430, dilution 1:20,000), goat anti-rabbit HRP (ThermoFisher Scientific: PIE31460, dilution 1:60,000) or goat anti-mouse IgM (Santa Cruz Biotechnology, Dallas, TX, USA: sc-2064, dilution 1:20,000). Specific immunoreactive bands were visualized by means of West Femto chemiluminescent reagents (ThermoFisher Scientific) using a Chemidoc MP with ImageLab software.

Statistical analysis

Data are given as the mean \pm SD unless otherwise stated. A Student's unpaired *t* test was used for comparison of two samples where data were normally distributed (Figs 3 and 6) and the Wilcoxin–Mann–Whitney test was used where data were not normally distributed (Fig. 4). Linear dependence between two variables examined by Pearson's correlation coefficient analysis (Fig. 5). *P* < 0.05 was considered statistically significant.

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 Ca²⁺ by exposure to the 'full release solution', containing 30 mM caffeine with low (0.05 mM) cytoplasmic Mg²⁺, and then reloaded with Ca²⁺ by exposure for a set time (10–180 s) to a load solution at pCa 6.7 before again emptying the SR (Fig. 1). The relative amount of Ca²⁺ accumulated by the SR could be gauged from the time-integral of the force response elicited in the full release solution, as previously described in detail (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 Mg²⁺. When the Ca²⁺ loading of the SR was carried out in the presence of 1 mM free Mg²⁺, which is close to the normal physiological level (Westerblad & Allen, 1992), the fibres from the Young subjects showed SR Ca²⁺ 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 \sim 22 s and 27 s in type I and type II fibres, respectively (Fig. 2B and Table 1). The relative amount of endogenous Ca²⁺ initially present in the SR, indicated by the force response upon first emptying the SR (Fig. 1) was \sim 52% and 40% of the maximal SR load level in those fibre types, respectively (Fig. 2B and Table 1), which also was very similar to that found previously in young subjects (Lamboley *et al.* 2013).

Although the characteristics of SR Ca²⁺ accumulation in fibres of the Old subjects were generally similar to those

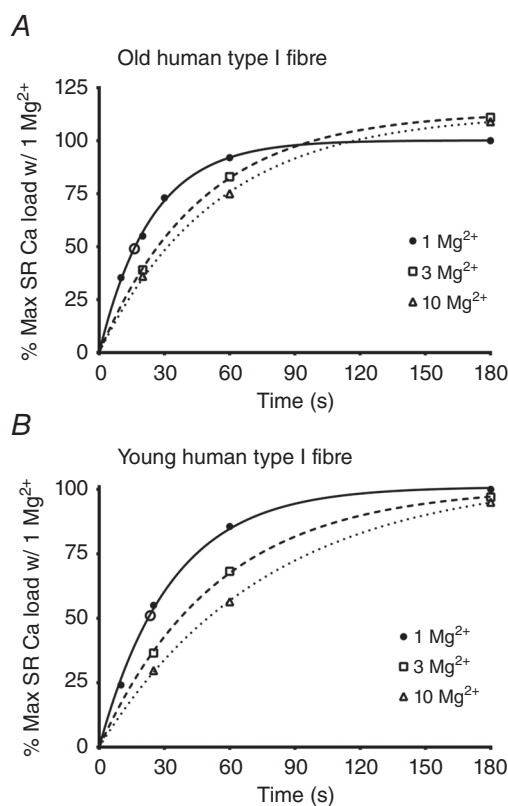


Figure 2. SR Ca²⁺ loading characteristics in type I fibre from an Old and a Young subject

Plots of relative SR Ca²⁺ content versus loading time when Ca²⁺ loading a type I fibre from an Old (A) or Young (B) subjects in the presence of 1, 3 or 10 mM Mg²⁺, derived from records in Figs 1A and 1B, respectively. Each value is expressed as a percentage of maximal SR Ca²⁺ capacity found with 180 s of loading in 1 mM Mg²⁺. Data sets fitted with best-fit single exponential function. Data have been adjusted for the negative ordinate intercept resulting from releasing SR Ca²⁺ in the presence of 0.5 mM EGTA (see Methods). Half-time for Ca²⁺ loading with 1 mM Mg²⁺ present was \sim 16 s and \sim 23 s in (A) and (B), respectively. Open circle symbols on fitted functions for 1 mM Mg²⁺ indicate the relative force–time integral found upon releasing the endogenous Ca²⁺ content, and correspond to \sim 49% and 51% of the maximum content in fibres in (A) and (B), respectively.

Table 1. SR Ca²⁺ loading properties of skinned muscle fibres from Young and Old subjects

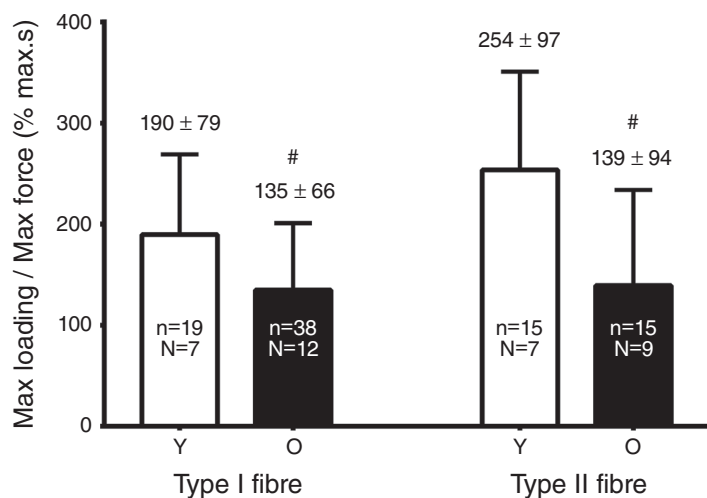
	Type I fibres		Type II fibres	
	Young	Old	Young	Old
Ca _{endo} (% maximum)	52 ± 9 (n = 25; N = 10)	55 ± 9 (n = 28; N = 7)	40 ± 7* (n = 30; N = 10)	40 ± 9* (n = 14; N = 8)
Half-time (s)	21.9 ± 7.9 (n = 34; N = 12)	18.7 ± 4.7 # (n = 39; N = 12)	26.8 ± 10.6* (n = 41; N = 13)	25.2 ± 6.6* (n = 14; N = 8)
% Full at 60 s of loading	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)

Mean ± SD of endogenous SR Ca²⁺ load level (Ca_{endo}) in mechanically skinned muscle fibres from Young and Old subjects, and half-time for SR Ca²⁺ loading in presence of 1 mM cytoplasmic Mg²⁺ (derived as in Figs 1 and 2). *n*, number of fibres; *N*, number of subjects. #Value in the Old group significantly different from the matching value in the Young group. *Value for type II fibres significantly different from that in type I fibres in same age group (Student's two-tailed *t* tests). Note that 60 s of loading time measurements were not made in some of the fibres for which loading curves were determined.

in Young subjects, one key difference was that Ca²⁺ loading in the type I fibres in the presence of 1 mM Mg²⁺ started to show saturation at relatively earlier loading times (e.g. compare the continuous line curves in Fig. 2A and B). 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 percentage filling of the SR with 60 s of loading (85.0 ± 6.1% versus 81.3 ± 6.1%, respectively, of that reached with 180 s of loading) (Table 1). Furthermore, it was apparent that the total amount of Ca²⁺ accumulated by the SR after 180 s of 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 Ca²⁺ (Fig. 3). Note that, in this assay, the time integral of the force response in each fibre was normalized to the maximal Ca²⁺-activated force measured in that fibre (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 accordance with our previous absolute measurements of SR Ca²⁺ content showing that the amount of releasable Ca²⁺ 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 Ca²⁺ (litre fibre volume)⁻¹, respectively] (Lamboley *et al.* 2015).

In type II fibres, there was no significant difference in either the half-loading time or the percentage SR filling at 60 s between Old and Young subjects (Table 1). Nevertheless, the time integral of the force response elicited upon releasing all SR Ca²⁺ in the type II fibres was substantially smaller (~55%) in the Old subjects compared to that in the Young subjects (Fig. 3). Some of this difference would have been caused by the lower Ca²⁺-sensitivity of the contractile apparatus in type II fibres in Old subjects relative to that in Young subjects (Lamboley *et al.* 2015), although it is nevertheless clear from our previous absolute measurements that

**Figure 3. Measure of maximum SR Ca²⁺ loading in 1 mM Mg²⁺ in fibres from Young (Y) and Old (O) subjects**

Mean ± SD of the time integral of the force response upon full release of SR Ca²⁺ after loading fibres for 180 s in the presence of 1 mM Mg²⁺ (Fig. 1); values normalized to maximum Ca²⁺-activated force measured in same fibre. *n*, number of fibres; *N*, number of subjects. #Value in the Old group significantly different from the matching value in the Young group (Student's paired two-tailed *t* test).

the maximal SR Ca²⁺ content of the type II fibres is significantly lower in Old compared to Young subjects [1.45 ± 0.11 versus 1.71 ± 0.11 mmol Ca²⁺ (litre fibre volume)⁻¹, respectively] (Lamboley *et al.* 2015).

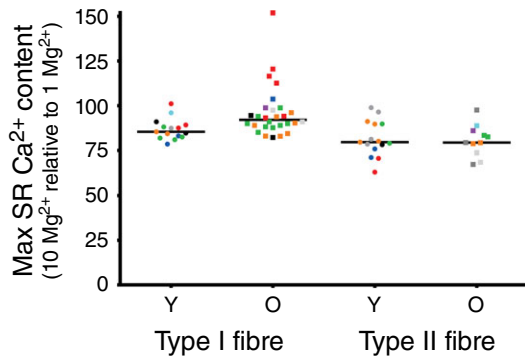


Figure 4. Effect of raised [Mg²⁺] on maximal SR Ca²⁺ accumulation in individual fibres

Values denote SR Ca²⁺ content accumulated after 180 s of loading in the presence of 10 mM cytoplasmic Mg²⁺, expressed as a percentage of that accumulated in the same fibre in 1 mM Mg²⁺, measured as in Figs 1 and 2. Each particular symbol labels fibres obtained from a given subject: nine Old (O) and nine Young (Y) subjects. Horizontal lines indicate median value in each case. The presence of 10 mM Mg²⁺ resulted in greater SR Ca²⁺ accumulation in type I fibres of Old subjects compared to that in type I fibres of Young subjects.

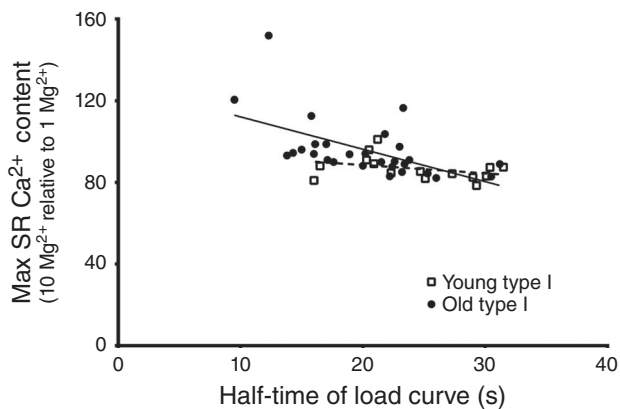


Figure 5. Inverse relationship between SR Ca²⁺ leak measure and half-time for SR Ca²⁺ accumulation in type I fibres of Old subjects

Relative SR Ca²⁺ accumulation in presence of 10 mM cytoplasmic Mg²⁺ (expressed as a percentage of that accumulated in 1 mM Mg²⁺) in individual type I fibres from Young and Old subjects versus half-time of SR Ca²⁺ loading in presence of 1 mM Mg²⁺ (measured as in Fig 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). The line of best fit is shown for each case.

Effect of raised cytosolic [Mg²⁺]

The relatively rapid saturation of the SR Ca²⁺ loading curve in the presence of 1 mM Mg²⁺, as well as the lower total SR Ca²⁺ accumulation, observed in the type I fibres of the Old subjects might be explained by increased leakage of Ca²⁺ out of the SR occurring in parallel with the uptake. To investigate whether there was increased Ca²⁺ leakage through the RyRs, we examined the effects of raising cytoplasmic [Mg²⁺] on the ability of the SR to accumulate Ca²⁺. Raising the free [Mg²⁺] from 1 mM to 3 or 10 mM slowed the rate of SR Ca²⁺ accumulation in fibres of both Young and Old subjects (Fig. 2), as expected from the known competitive effects of Mg²⁺ on Ca²⁺ pumping by sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) (Chiesi & Inesi, 1981; Kabbara & Stephenson, 1994), although the fibres were still able to accumulate substantial amounts of Ca²⁺ provided that the loading time was sufficiently long. In the type I fibres of Young subjects, the amount of Ca²⁺ accumulated by the SR after 180 s of loading in the presence of 10 mM Mg²⁺ reached an average of ~87% of that accumulated in 1 mM Mg²⁺ (Fig. 2); the individual values found in 15 type I fibres from seven Young subjects are shown in Fig. 4. In comparison, the type I fibres from Old subjects in the presence of 10 mM Mg²⁺ accumulated an average of ~96% of that accumulated in 1 mM Mg²⁺ and, strikingly, a number of the fibres accumulated more total Ca²⁺ in the SR when loaded in 10 mM Mg²⁺ than in 1 mM Mg²⁺ (e.g. Figs 2A and 4, showing 28 type I fibres from seven Old subjects).

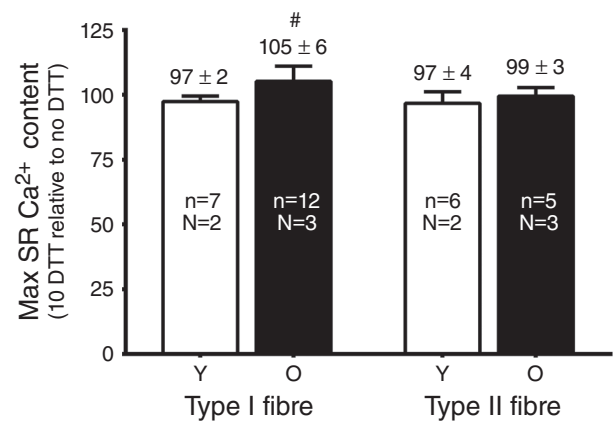


Figure 6. Effect of reducing treatment (10 mM DTT) on SR Ca²⁺ accumulation

Mean ± SD of maximum SR Ca²⁺ accumulation (180 s of loading time) in 1 mM Mg²⁺ 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 , number of fibres; N , number of subjects. [#]Value significantly greater than 100% ($P < 0.025$, Student's one-tailed t test). Note that a small decrease in the amount of Ca²⁺ accumulation is observed upon successive measurements even without DTT treatment.

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; a non-parametric test is used because values in Old subjects were not normally distributed). Furthermore, analysing 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 ($P = 0.013$, seven Old *versus* seven Young subjects, Wilcoxin–Mann–Whitney test). It was also apparent that the type I fibres of the Old subjects with the 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+} (Fig. 5), which is consistent with the saturating behaviour of the Ca^{2+} loading being the result of increased SR Ca^{2+} leakage through the RyRs. Taken together, these data show that raising the cytosolic $[\text{Mg}^{2+}]$ 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. By contrast, in type II fibres, the effect of raising cytosolic $[\text{Mg}^{2+}]$ on SR Ca^{2+} accumulation was not noticeably different between Old and Young subjects (Fig. 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 of loading in the presence of 1 mM Mg^{2+} was measured both before and after 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 the successive load–release cycle [percentage accumulation on second control trial relative to first: $98 \pm 2\%$ in Young type I ($n = 7$); $97 \pm 5\%$ Old type I ($n = 23$); $94 \pm 6\%$ in Young type II ($n = 9$); $99 \pm 2\%$ in Old type II fibres].

Densities of DHPRs and RyR1s

We used western blotting to examine the densities of the DHPRs ($\alpha 1$ 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 (Fig. 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).

Table 2. DHPR and RyR densities in muscle homogenates of Young and Old subjects

	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

Mean \pm SD relative density of DHPRs and RyR1s, obtained by western blotting, as in Fig. 7. Values for each subject normalized to the average of that found in the samples of all Young subjects on the same gel. Values are the average of duplicate measurements from different gels.

We also examined the DHPR and RyR1 densities in individual muscle fibres (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 both the DHPRs and the RyR1s were $\sim 50\%$ higher in the type II fibres ($n = 19$) than in the type I fibres ($n = 17$) on average. 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 $61 \pm 19\%$ and $114 \pm 33\%$, respectively, of that found in the muscle of the Young subjects; these values primarily reflected the decreased proportion of type II fibres and the increased proportion of type I fibres in the muscle of the Old subjects (Lamboley *et al.* 2015). The relative density of SERCA2 found in six type I fibres from four Old subjects was an average of $94 \pm 24\%$ of that found in five 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

The present 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

and 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 Ca²⁺ leakage in the former. Importantly, raising the cytoplasmic [Mg²⁺] from 1 to 10 mM, aiming to specifically decrease Ca²⁺ leakage through the RyRs (Meissner *et al.* 1986; Lamb *et al.* 2001; Laver *et al.* 2004), was found to increase net SR Ca²⁺ 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 accumulating more total SR Ca²⁺ in 180 s of loading in 10 mM Mg²⁺ than in 1 mM Mg²⁺ despite the strong competitive effects of the increased [Mg²⁺] on SERCA Ca²⁺ pumping (Chiesi & Inesi, 1981; Kabbara & Stephenson, 1994). Furthermore, treatment with the reducing agent DTT also increased net Ca²⁺ 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 [Mg²⁺] (Fig. 4). We have shown previously in rat muscle fibres that oxidative treatment with the sulphhydryl-specific oxidant 2,2'-dithiodipyridine causes increased Ca²⁺ leakage from the SR and that reducing treatment with DTT reverses this effect (Posterino & Lamb, 1996). The above data demonstrate that: (i) there is increased Ca²⁺ 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) the leakage occurs primarily through the RyRs; and (iii) strong reducing treatment inhibits this RyR Ca²⁺ leakage, increasing net SR Ca²⁺ accumulation. These findings are analogous to those made in the muscle of aged mice (in muscles with predominantly type II fibres), where there was increased oxidation/nitrosylation of the RyRs, leading to greater SR Ca²⁺ leak and decreased SR Ca²⁺ content, which could be reversed by *in vitro* treatment with DTT (see Introduction) (Andersson *et al.* 2011; Umanskaya *et al.* 2014).

The present study did not find evidence of increased Ca²⁺ leakage in the type II fibres of the Old subjects; specifically, there was no significant difference from the type II fibres of Young subjects with regard to the time course of Ca²⁺ accumulation (Table 1), the effect of raising [Mg²⁺] to 10 mM (Fig. 4) or the effect of DTT treatment (Fig. 6). Nevertheless, we have previously observed that the maximal level of SR Ca²⁺ 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 Ca²⁺ accumulation assay used in the present study is a relatively insensitive measure of SR Ca²⁺ leakage because the [Ca²⁺] within the skinned fibres

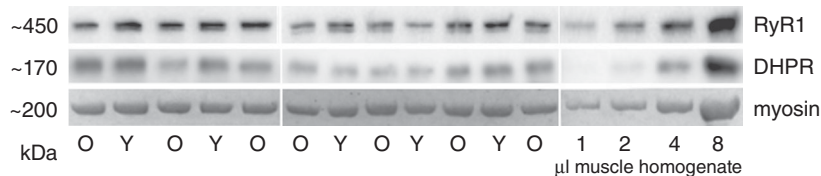


Figure 7. DHPR and RyR density in muscle homogenates from Old and Young subjects

Upper panels: western blots for DHPR α 1 subunit and RyR1 in muscle homogenates from seven Old (O) and five Young (Y) subjects. Right-hand lanes contained 1–8 μ l 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.

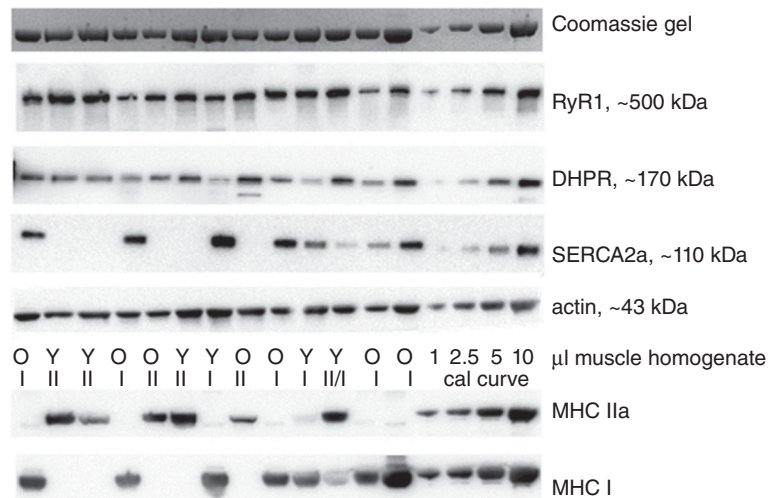


Figure 8. DHPR and RyR density in single muscle fibres from Old and Young subjects

Western blots for DHPR α 1, 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 1–10 μ l 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: myosin band on Coomassie-stained post-transfer gel.

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 the 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, possibly in part because the SERCA density in the type II fibres (almost exclusively SERCA1a; Lamboley *et al.* 2014) is probably substantially higher than in type I fibres (almost exclusively SERCA2a), assuming that human fibres show a relative SERCA density distribution similar to that seen in rat and rabbit muscle (Leberer & Pette, 1986; Wu & Lytton, 1993). The level of Ca^{2+} buffering used in the assay of the present study 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, at least in the type I fibres of the Old subjects, there is substantially increased Ca^{2+} leakage through the RyRs resulting from reversible oxidative modification of cysteine residues on the RyRs because this leakage 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 probably all reflected irreversible oxidative damage of the contractile apparatus. It was noted that type I fibres were probably less susceptible to such irreversible oxidative damage than type II fibres because type I fibres contain higher levels of glutathione (GSH) and superoxide dismutase (at least in rats) (Ji *et al.* 1992; Masuda *et al.* 2003). This may well also help explain 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 not only 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 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), and which would increase resting Ca^{2+} leakage. Furthermore, there would probably 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), and this 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 could be a result of 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 a result of the modification of only a small proportion of the total pool of RyRs. We acknowledge that this is a limitation of the present study and note that a more complete understanding of the basis of the oxidation-linked increase in SR Ca^{2+} leakage in ageing muscle will probably require a range of mass-spectroscopic and labelling studies and further advances in knowledge about the effects of specific oxidative changes on the 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 illustrated by studies on single muscle fibres in which increased expression of superoxide dismutase or application of various different combinations of ROS/RNS-neutralizing compounds was found to differentially alter 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 α 1 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 a 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 in the present study, 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; Lambolley *et al.* 2015) and the fact that the density of both DHPRs and RyRs is ~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 in the present study from a subset of the subjects, the DHPR density was, if anything, somewhat higher in the fibres of the Old subjects.

Conclusions

The findings obtained in the present study demonstrate that there is increased leakage of Ca²⁺ out of the SR through the RyRs in type I muscle fibres in aged humans as a result of the reversible oxidative modification of the RyRs, and that this Ca²⁺ leakage is probably the primary cause of the decreased SR Ca²⁺ content seen in such fibres (Lambolley *et al.* 2015). Such SR Ca²⁺ 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 may arise from a self-reinforcing cycle in which Ca²⁺ leakage through the RyRs leads to increased ROS production by the mitochondria, which in turn further exacerbates RyR leakage. The increase in local [Ca²⁺] within the triad junction may also contribute to the decreased SR Ca²⁺ release observed in muscle of aged humans and rodents (Delbono *et al.* 1995; Wang *et al.* 2000) by increasing the activity of Ca²⁺-dependent proteases (calpains), leading to disruption of the normal coupling between the DHPRs and the RyRs (Lamb *et al.* 1995; Murphy *et al.* 2013). Taken together with the direct effects of the SR Ca²⁺ depletion on Ca²⁺ release (Posterino & Lamb, 2003), this 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 (Lambolley *et al.* 2015).

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

Muscle biopsies were performed at Victoria University. Biochemical and physiological measurements on skinned fibres were conducted 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. RMM and GDL were responsible for western blotting procedures and analysis, with the assistance of VLW. CRL and GDL drafted the manuscript. All authors were involved in the conception of the project. All authors have

approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

Supported by National Health & Medical Research Council of Australia (Grant number 1051460).

Acknowledgements

We thank Maria Cellini and Heidy Latchman for technical assistance. We also thank Dr Mitchell Anderson for performing muscle biopsies. The monoclonal antibodies directed against adult human MHC isoforms (A4.840 and A4.74) used in the present study were developed by Dr H. Blau; those directed against SERCA1 were developed by Dr D. Fambrough; those directed against RyR1 were developed by Drs J. Airey and J. Sutko; and those directed against DHPR were developed by Dr K. P. Campbell. All were obtained from the Development Studies Hybridoma Bank, under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.