Resistance training-induced changes in integrated myofibrillar protein synthesis are related to hypertrophy only after attenuation of muscle damage

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Key points

- Skeletal muscle hypertrophy is one of the main outcomes from resistance training (RT), but how it is modulated throughout training is still unknown.
- We show that changes in myofibrillar protein synthesis (MyoPS) after an initial resistance exercise (RE) bout in the first week of RT (T1) were greater than those seen post-RE at the third (T2) and tenth week (T3) of RT, with values being similar at T2 and T3.
- Muscle damage (Z-band streaming) was the highest during post-RE recovery at T1, lower at T2 and minimal at T3.
- When muscle damage was the highest, so was the integrated MyoPS (at T1), but neither were related to hypertrophy; however, integrated MyoPS at T2 and T3 were correlated with hypertrophy.
- We conclude that muscle hypertrophy is the result of accumulated intermittent increases in MyoPS mainly after a progressive attenuation of muscle damage.

Abstract Skeletal muscle hypertrophy is one of the main outcomes of resistance training (RT), but how hypertrophy is modulated and the mechanisms regulating it are still unknown. To investigate how muscle hypertrophy is modulated through RT, we measured day-to-day integrated myofibrillar protein synthesis (MyoPS) using deuterium oxide and assessed muscle damage at the beginning (T1), at 3 weeks (T2) and at 10 weeks of RT (T3). Ten young men (27 (1) years, mean (SEM)) had muscle biopsies (vastus lateralis) taken to measure integrated MyoPS and muscle damage (Z-band streaming and indirect parameters) before, and 24 h and 48 h post resistance exercise (post-RE) at T1, T2 and T3. Fibre cross-sectional area (fCSA) was evaluated using biopsies at T1, T2 and T3. Increases in fCSA were observed only at T3 ($P = 0.017$). Changes in MyoPS post-RE at T1, T2 and T3 were greater at T1 ($P < 0.03$) than at T2 and T3 (similar values between T2 and T3). Muscle damage was the highest during post-RE recovery at T1, attenuated at T2 and further attenuated at T3. The change in MyoPS post-RE at both T2 and T3, but not at T1, was strongly correlated ($r \approx 0.9$, $P < 0.04$) with muscle hypertrophy. Initial MyoPS response post-RE in an RT programme is not directed to support muscle hypertrophy, coinciding with the greatest muscle damage. However, integrated MyoPS is quickly 'refined' by 3 weeks of RT, and is related to muscle hypertrophy. We conclude that muscle hypertrophy is the result of accumulated intermittent changes in MyoPS post-RE in RT, which coincides with progressive attenuation of muscle damage.

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Abbreviations   CK, creatine kinase; D_2O, deuterium oxide; CSA, cross-sectional area; fCSA, fibre cross-sectional area; FSR, fractional synthetic rate; MVC, maximum voluntary isometric torque; MyoPS, myofibrillar protein synthesis; RE, resistance exercise; RT, resistance training; SOR, muscle soreness; T1, first week of training; T2, third week of training; T3, tenth week of training; VL, vastus lateralis.

Introduction

Increases in skeletal muscle cross-sectional area (CSA) (i.e. muscle hypertrophy) are a hallmark adaptation of resistance training (RT). The processes modulating muscle hypertrophy during RT are still surprisingly obscure (Phillips, 2012; Schoenfeld, 2012; Damas *et al.* 2016*a,b*). RT-induced muscle hypertrophy is the result of an accumulation of intermittent increases in myofibrillar muscle protein synthesis (MyoPS) in response to each resistance exercise (RE) bout (Moore *et al.* 2009*b*; Brook *et al.* 2015). Indeed, we had previously reported that acute increases in MyoPS aligned qualitatively with hypertrophy-related chronic RT outcomes, such as increases in muscle volume and muscle fibre CSA (fCSA) (West *et al.* 2009, 2010; Burd *et al.* 2010; Mitchell *et al.* 2012). Conversely, we (Mitchell *et al.* 2014) and others (Mayhew *et al.* 2009) have reported that the increases in protein synthesis after an initial RE bout, measured by infusion trials over hours, did not align with the eventual muscle hypertrophy after RT. The reason for the incongruence between acute and chronic hypertrophy-related responses is unknown, but could be due to a physiological mechanism or a methodological issue of evaluating protein synthesis over a short time period (Mitchell *et al.* 2014).

Physiologically, unaccustomed RE results in muscle damage evidenced directly by assessing muscle ultrastructure changes (e.g. Z-band streaming) (Gibala *et al.* 1995; Stupka *et al.* 2001; Beaton *et al.* 2002*b*), and indirectly by proxy indices (e.g. loss of muscle strength, increases in muscle soreness (SOR) and creatine kinase (CK) activity in the blood) (Clarkson *et al.* 1992; Conceição *et al.* 2012; Nogueira *et al.* 2014). After the initial muscle damage, growth mechanisms are triggered (Vierck *et al.* 2000; McKay *et al.* 2008; Mackey *et al.* 2011) producing an increase in protein synthesis to support, at least in part, tissue repair (Moore *et al.* 2005). Thus, the lack of correlation between the initial post-RE increase in protein synthesis and chronic muscle hypertrophy (Mitchell *et al.* 2014) could be due to damage to protein structures that would require repair, and therefore a greater increase in protein synthetic response. This large protein synthetic response has led researchers to propose that muscle damage is concomitant with and a prerequisite for muscle hypertrophy (Moore *et al.* 2005; Shepstone *et al.* 2005). However, muscle damage is rapidly attenuated (i.e. the repeated bout effect) (Clarkson & Hubal, 2002; Chen *et al.* 2009), raising uncertainty as to the role of muscle damage *per se* in long-term RT-induced muscle hypertrophy. Additionally, regular RT modifies the protein synthetic response to become much shorter in duration after each RE bout (Phillips *et al.* 1999, 2002; Kim *et al.* 2005; Tang *et al.* 2008). Considering that the initial acute protein synthetic response does not correlate with muscle hypertrophy (Mitchell *et al.* 2014), and the pattern of increase in protein synthetic responses is modulated with RT, it seems plausible that at later times during the course of RT there is an attenuation and 'refinement' of the protein synthetic response (Wilkinson *et al.* 2008), which would result in the protein synthetic response being directed less toward repair of muscle damage and more toward muscle fibre hypertrophy.

Methodologically, the use of deuterium oxide (D_2O) ingestion to evaluate MyoPS allows capturing variations in the protein synthetic response over days to weeks, thus enabling a more integrative measure not seen in the hour-to-hour method of tracer infusion. For example, Brook *et al.* (2015) showed that MyoPS measured by D_2O ingestion over a 3 week period (RT three times weekly) was related to the extent of increase in muscle thickness seen with RT. A subsequent 3 week period (weeks 3–6) of RT showed that integrated MyoPS had returned to basal levels and no further relationship with increase in muscle thickness was found. The return of MyoPS rates to baseline after only 3 weeks of RT observed by Brook *et al.* (2015) is in contrast to multiple studies that have shown that, even in the trained state, individuals mount a robust acute (hour-to-hour) protein synthetic response post-RE with or without nutrition (for review see Damas *et al.* 2015). We propose that measuring MyoPS on the days following RE, rather than over weeks, would provide a more refined estimation of the contribution of MyoPS to hypertrophy.

We investigated how muscle hypertrophy was modulated throughout RT assessing MyoPS and muscle damage at the first week (T1), 3 weeks into training (T2), and at the end of a 10 week RT (T3). MyoPS was measured using an integrated approach by D_2O ingestion (MacDonald *et al.* 2013; Wilkinson *et al.* 2014; Brook *et al.* 2015) on a day-to-day basis after RE with the rationale that this would capture an acute but integrated response over 48 h post-RE. We hypothesized that MyoPS would be

highest at T1, concomitant with highest muscle damage, but that MyoPS would be attenuated at T2 and T3. We also hypothesized that muscle damage would be progressively attenuated throughout the RT programme. Finally, we hypothesized that the initial MyoPS responses would not be related to muscle hypertrophy; however, integrated MyoPS would be related to muscle hypertrophy at T2 and T3.

Methods

Participants

The present study was approved by The Human Research Ethics Committee of the University of São Paulo (no. 608.754, 27 March 2014) and all procedures performed were in accordance with the *Declaration of Helsinki*. All individuals signed an informed consent form before participation. A total of 10 healthy young men (age 27 (1) years, mean (SEM); body mass index 23.6 (1.0) kg m^{-2}; complete participants' characteristics are published elsewhere, Damas *et al.* 2016a) with previous experience in lower limb RT, but who had not engaged in lower limb RT for at least 6 months prior to the study and did not use vitamin supplements or anti-inflammatory medications chronically, were recruited.

General design

A schematic illustration of the experimental design is shown in Fig. 1. Participants performed RT for their lower limbs (bilateral 45 deg leg-press exercise and leg extension)

for 10 weeks (twice a week, totalling 19 workouts). We studied the participants at three training phases during the RT programme: T1 (at baseline and after their initial workout), T2 (at 3 weeks of RT, before and after the fifth workout) and T3 (at 10 weeks of RT, before and after the 19th workout). Muscle samples were collected through biopsies and used to analyse fCSA, MyoPS and Z-band integrity. At T1, muscle samples were collected 1 day before RE (−24 h), immediately pre-RE (0 h) and 24 h and 48 h post-RE. At T2 and T3, muscle biopsies were collected at immediately pre-RE (0 h) and 24 h and 48 h post-RE. fCSAs were assessed using 0 h biopsies at T1, T2 and T3. Regarding MyoPS, at T1, we could calculate values for resting MyoPS (difference between 0 h and −24 h), 24 h (difference between 24 h post-RE and 0 h), 48 h post-RE (difference between 48 h post-RE and 24 h post-RE) and the integrated first 48 h post-RE (difference between 48 h post-RE and 0 h). For T2 and T3, we could only calculate MyoPS 24 h and 48 h post-RE, and integrated first 48 h post-RE. Two days before the first biopsy in each evaluation week, participants ingested a bolus of 100 ml of 70% deuterated water (D$_2$O, Cambridge Isotope Laboratories, Tewksbury, MA, USA) for the MyoPS analysis, and saliva samples were collected daily to measure the decay of D$_2$O in total body water. For Z-band streaming, we used 0 h and 48 h biopsies at T1, T2 and T3. In addition, we evaluated maximum voluntary isometric contraction torque (MVC) and SOR before and 24 h and 48 h post-RE at T1, T2 and T3. Blood samples (4 ml) were collected before and 48 h post-RE for analysis of CK activity.
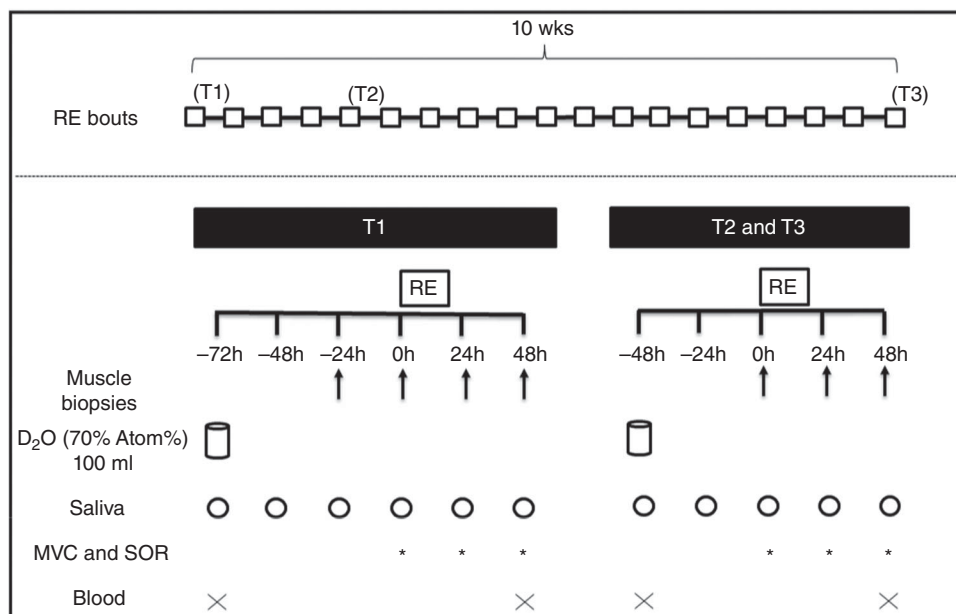

Figure 1. Experimental design
RE: resistance exercise; D$_2$O: deuterated water; MVC: maximal voluntary isometric torque; SOR: muscle soreness; T1: first week of resistance training; T2: third week of resistance training; T3: last week of resistance training.

Diet and physical activity

Three days before the beginning of the study and throughout the study period participants were instructed to maintain their normal eating habits, to not consume supplements (except those we provided), and to refrain from other physical activities. Immediately after every RE bout throughout the RT period each participant was provided with 25 g of whey protein in order to maximally stimulate MyoPS (Tipton *et al.* 1999; Moore *et al.* 2009*a*; Cermak *et al.* 2012). Importantly, the diet in the days of biopsies was standardized (22% protein, 41% carbohydrates and 37% lipids).

Resistance training

The RE protocol was composed of 45 deg inclined leg-press and leg extension exercises (3 sets each, 9–12 maximum bilateral repetitions per set, 90 s rest between sets), with load adjustments through sets and sessions to maintain the same repetition range (Damas *et al.* 2016*a*). Importantly, every set was performed to volitional fatigue.

Muscle biopsy

Muscle samples from the vastus lateralis were obtained through percutaneous muscle biopsy with manual suction. Sequential samples were taken from alternate legs; however, the pre-RE biopsies were always performed in the same leg at T1, T2 and T3. This procedure was conducted so the same sequence of legs were biopsied at T1, T2 and T3 and thus, reducing variability among evaluation weeks. The biopsy procedure involved administration of local anaesthesia (2–3 ml of 1% Xylocaine (lignocaine)) and via a small incision ~100 mg of muscle was collected. The tissue was dissected free from blood and connective tissue. A small portion (20–30 mg) from the biopsies obtained before the RE at T1, T2 and T3 was placed in optimum cutting temperature (OCT) embedding medium with its fibres perpendicular to the horizontal surface and quick-frozen in isopentane cooled by liquid nitrogen to perform fCSA analysis. A larger portion (40–50 mg) was used to quantify the rate of MyoPS in all of the muscle specimens collected. Finally, 5–10 mg from the biopsies obtained before and at 48 h post-RE were placed in a fixative (2% gluteraldehyde) for staining with toluidine blue for further light microscopy analysis. After separation in aliquots, all tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Fibre cross-sectional area (fCSA)

Muscle samples were sectioned at 7 μm, brought to room temperature and fixed in 2% paraformaldehyde (PFA) for 10 min, washed and then blocked for 90 min in PBS (containing 2% bovine serum albumin (BSA), 5% fetal bovine serum, 0.02% Triton X-100, 0.1% sodium azide and 5% goat serum). Subsequently, samples were incubated overnight with primary antibody (rabbit, Abcam, Toronto, ON, Canada) against laminin (1:500) diluted in 1% BSA at 4°C. The next day, slides were incubated with the secondary antibody (Alexa Fluor 488-R, Invitrogen, Toronto, ON, Canada) at 1:500 in 1% BSA in the dark for 2 h at room temperature. Samples were then re-washed and coverslipped. Images were observed and captured using the Nikon Eclipse 90i microscope at a magnification of ×20 and the Photometrics Cool SNAP HQ2 fluorescent camera (Nikon Instruments, Melville, NY, USA), respectively. Fibres were then delineated using the Nikon NIS-Elements AR software (Nikon Instruments) on a large-scale image. A mean of 80 fibres were analysed per time point by the same investigator in a blinded fashion. The reliability between two blinded measurements was good (typical error = 2.1%).

Integrated muscle protein fractional synthetic rate

To determine the MyoPS rate, we used the ingestion of deuterated water (D_2O) that has been used previously to measure protein fractional synthetic rate (FSR) in humans (Gasier *et al.* 2012; MacDonald *et al.* 2013; Wilkinson *et al.* 2014; Bell *et al.* 2015). D_2O was given to the participants as a bolus (100 ml of 70% D_2O) 48 h before the first biopsy at T1, T2 and T3. The enrichment of body water with D_2O was assessed through daily saliva samples.

Muscle samples were prepared (isolation, hydrolysing, purification and derivatization of the myofibrillar proteins) following procedures described by Moore *et al.* (2009*b*) and Burd *et al.* (2012). Briefly, a portion of muscle (40–50 mg) was homogenized on ice in buffer (10 μl mg^{-1} 25 mM Tris, 0.5% v/v Triton X-100 and protease/phosphatase inhibitor cocktail tablets (Complete Protease Inhibitor Mini-Tabs, Roche, Indianapolis, IN, USA; PhosSTOP, Roche Applied Science, Mannhein, Germany)). Samples were then centrifuged at 1500 *g* for 10 min at 4°C to separate the supernatant (sarcoplasmic) and pellet (myofibrillar) fractions. The myofibrillar fraction was stored at −80°C for future processing. The myofibrillar pellet was solubilized in 1 ml NaOH (0.3 M) and heated to 50°C for 30 min with vortex mixing every 10 min. Samples were centrifuged at 4000 *g* for 5 min at 4°C, the supernatant containing the myofibrillar fraction was collected and the collagen pellets discarded. Myofibrillar proteins were precipitated by adding 1 ml of 1 M perchloric acid (PCA) and spinning at 700 *g* for 10 min at 4°C. The supernatant was discarded and the pellet was washed using 1 ml of 70% ethanol. Amino acids from myofibrillar proteins were released using acid hydrolysis by incubating in 1 ml HCl (1 M) and

1 ml of Dowex resin (50WX8-200 resin; Sigma-Aldrich) with HCl (0.5 M) and heated to 100°C for 72 h with vortex mixing every 24 h. Samples were eluted from the resin with 2 M NH$_4$OH and evaporated to dryness. Muscle preparations were analysed by Metabolic Solutions for incorporation of deuterated alanine with a Thermo Finnigan Delta V IRMS coupled to a Thermo Trace GC Ultra with a GC pyrolysis interface III and Conflow IV (gas chromatograph (GC)-pyrolysis-isotope ratio mass spectrometer (IRMS)) (Metabolic Solutions, Nashua, NH, USA). The *N*-acetyl-n-propyl ester of alanine was analysed using a splitless injection with a CTC Pal auto-sampler (1 μl), at an injection temperature of 250°C, and using a Zebron ZB-5 column of 30 m × 0.25 mm × 0.50 μm film thickness (Phenomenex, Torrance, CA, USA). The GC oven was programmed with an initial column temperature of 80°C with a 2 min hold, followed by a ramp of 30°C per minute up to 330°C. Compounds eluting off the column were directed into the pyrolysis reactor, heated at 1450°C and converted to hydrogen gas. The deuterated enrichment was first initially expressed in δ values compared to a calibrated hydrogen gas and then converted to atom% by standard equations. Methylpalmitate (courtesy of Dr Arndt Schimmelmann, Biogeochemical Laboratories, Indiana University) was used as the calibration standard.

Saliva samples were analysed for deuterium enrichment by cavity ring-down spectroscopy using a Liquid Water Isotope Analyser with automated injection system, version 2 upgrade (Los Gatos Research, Mountain View, CA, USA). Samples were vortexed and spun at 2000 g to remove any particulates. The water phase of saliva was injected 6 times and the average of the last three measurements used for data analysis. A standard curve was run before and after samples for calculation of deuterium enrichment. Intra-run precision was less than 2δ per millilitre (parts per thousand) and inter-run precision was less than 3.5δ per millilitre.

Myofibrillar protein fractional synthetic rate (FSR, % day^{-1}) was determined from the incorporation of deuterium-labelled alanine into protein, using the enrichment of body water (corrected for the mean number of deuterium moieties incorporated per alanine, 3.7, and the dilution from the total number of hydrogens in the derivative, 11) as the surrogate precursor labelling between subsequent biopsies. Therefore, the following formula was used:

$$\text{FSR}\left(\frac{\%}{\text{d}}\right) = \frac{(\text{APE}_{\text{Ala}}/\text{APE}_{\text{p}})}{\text{t} \times 100}$$

where APE_{Ala} is the deuterium enrichment of protein-bound alanine (in atom% excess), APE_{p} is the mean deuterium enrichment (in atom% excess) in total body water between the time points, and *t* is the time (in days) between biopsies.

Toluidine blue-stained light microscopy

Tissue samples were fixed in osmium tetroxide, dehydrated in graded ethanol baths and embedded in epoxy resin (Spurr's) with fibres oriented longitudinally. Each block was sectioned (0.5 mm) and marked with toluidine blue. Individual fibres for each section of longitudinal muscle were examined at ×1000 magnification in a light microscope for areas of Z-band streaming. In a pilot experiment, we selected sections identified to be damaged using toluidine blue staining/light microscopy and confirmed the disruption with ultrathin sections from the same blocks under electron microscopy examination; in this experiment there was excellent inter-rater agreement (intraclass correlation coefficient 0.97) on damaged sections. Z-band streaming was scored by two methods always using longitudinal cuts. First, the total visible fibre area and Z-band streaming area were calculated in each image and percentage area occupied by Z-band streaming per total fibre area analysed was recorded. Second, the number of fibres that showed a visible area of Z-band streaming was counted and the percentage of affected fibres per total number of fibres analysed was determined. We analysed the images acknowledging if we were analysing the same fibre as in the previous image or a new fibre. Several times, different parts of the same fibre were analysed in more than one image. The mean number (SD) of fibres analysed per subject per time point was: 23 (15), range 10–60, and the number of images analysed was 28 (20), range 10–96. Figure 2 depicts an example of a normal (non-affected) fibre (i.e. no Z-band streaming; Fig. 2*A*) and a fibre exhibiting Z-band streaming (Fig. 2*B*). With the 0 h biopsy (i.e. pre-RE) sample as the basis of comparison, we ensured that no damage had occurred as a result of the biopsy procedure. The same investigator analysed all images in a blinded fashion and the reliability between two blinded measurements was very good (typical error = 1%).

Indirect markers of muscle damage (MVC, SOR and plasma CK activity)

Participants were evaluated for their knee extension MVC in an isokinetic dynamometer (Biodex System Pro 4, Biodex Medical Systems, Shirley, NY, USA). Extraneous movements were restrained by straps across their chest, waist and thigh of their dominant leg. The hip joint was fixed at 85 deg of flexion and arms were folded across their chest. A specific warm-up composed of two sets of three knee extensions/flexions contractions (90 deg s^{-1}; 90 deg of range of motion) separated by 30 s rest interval was performed before measurements. After 60 s, the participants performed three knee extension MVC attempts (5 s duration) at 60 deg of knee extension to the horizontal, separated by

60 s rest intervals (Damas *et al.* 2016*a*). The highest peak torque obtained among the three attempts was considered as the MVC value. All participants were verbally encouraged to contract as hard as possible. The reliability between two repeated measures was good (typical error = 3.6%).

Perceived SOR was assessed using a 100 mm visual analog scale (0 mm: 'not sore at all', 100 mm: 'very, very sore') (Nogueira *et al.* 2014). Participants assessed their SOR by placing a mark on the scale after sitting and rising from a chair without any help of the arms two times. The distance in millimetres between zero and the mark on the scale was used for subsequent analyses.

Blood samples (4 ml) were drawn from an antecubital vein for CK analysis. Samples were centrifuged for 10 min to obtain plasma and stored at $-80°C$. CK activity was measured using a colorimetric kit (Sigma, MAK116; Oakville, ON, Canada). The normal reference range for CK activity using this method is 20–180 U l^{-1}. The intra-assay coefficient of variation on 10 repeat samples was <5%.

Statistical analysis

To test for the effects of RT in fCSA and the change (0–48 h) in the dependent variables, a mixed model analysis was used having training phase (T1, T2 and T3) as fixed factor and subjects as a random factor. To analyse the changes in the change in MyoPS two procedures were conducted. Firstly, we tested if MyoPS 24 h and 48 h post-RE at T1, T2 and T3 were different from resting MyoPS (-24 h to 0 h) at T1 (i.e. control condition) using a Dunnett's test. Secondly, to investigate if changes in MyoPS 24 h and 48 h post-RE were different among training phases we applied a mixed model assuming acute time changes (24 h and

48 h) and training phase (T1, T2 and T3) as fixed factors and subjects as a random factor. To analyse differences in the changes in indirect markers of muscle damage over time at different training phases a mixed model assuming time and training phase as fixed factors and subjects as a random factor was used. In all models when a significant *F* value was obtained, a Fisher's LSD was used for pair-wise comparisons. We performed a series of correlations using the Pearson's product moment correlation between hypertrophic variables (MyoPS, fCSA and vastus lateralis (VL) CSA (VL CSA data from Damas *et al.* 2016*a*)) and muscle damage variables (Z-band streaming, MVC, SOR and CK), and between acute measurements of hypertrophic responses (MyoPS) and chronic hypertrophic outcomes (fCSA and VL CSA). Correlation coefficients $|r| < 0.2$ were considered small; $0.2 < |r| < 0.7$, moderate; and $|r| > 0.7$, high. Significance level was set as $P \leq 0.05$. Data are presented as means (SEM) unless otherwise stated.

Results

Fibre cross-sectional area

Mean fCSA increased significantly only at T3 compared with T1 ($P = 0.017$) and T2 ($P = 0.027$; Fig. 3).

Myofibrillar muscle protein synthesis

Figure 4 depicts mean body water enrichment (atom% excess (APE)) over the study period. After a 100 ml D_2O bolus at -48 h T1, and -24 h T2 and T3, body water enrichment depicts a linear decay over time (r^2 for linear regression at T1, T2 and T3 were 0.981, 0.997 and 0.999, respectively).
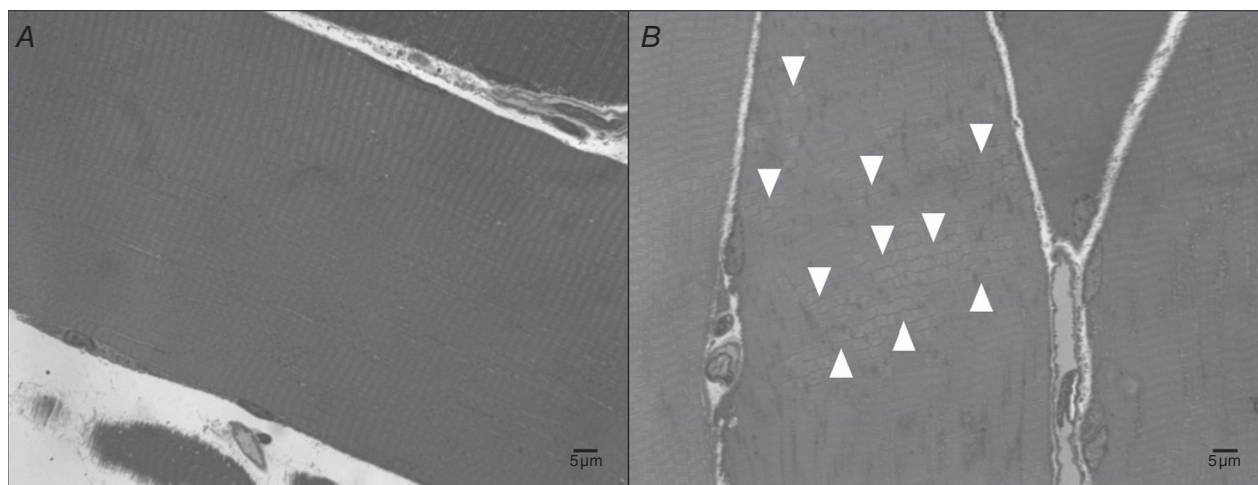

Figure 2. Muscle damage
Toluidine blue staining of muscle fibres showing (*A*) a fibre with no Z-band streaming and (*B*) a fibre with areas (white arrows) of Z-band streaming.

Figure 5*A* shows that MyoPS increased significantly 24 h and 48 h T1, 24 h T2 and 24 h T3 compared to resting value at T1 ($P \leq 0.05$). There were greater changes in MyoPS at T1 than at both T2 and T3 ($P < 0.03$), but similar changes were found at T2 and T3 ($P = 0.748$). In addition, MyoPS values 24 h were higher than 48 h independent of training phase ($P = 0.003$). Integrated MyoPS over the first 48 h (0–48 h) after RE indicated higher values for T1 compared with T2 and T3 ($P < 0.03$) (values at T2 and T3 were similar, $P = 0.8$) (Fig. 5*B*). In an attempt to account for the amount of MyoPS required only to repair (not induce hypertrophy) the 'damaged' area we corrected the MyoPS for the Z-band streaming area (using the first method for Z-band streaming calculation that considers the 'area' explained in the Methods) using the formulae: MyoPS × (100 − Z-band streaming)/100. After the correction, no differences were found between T1, T2 and T3 ($P > 0.05$; Fig. 5*C*).
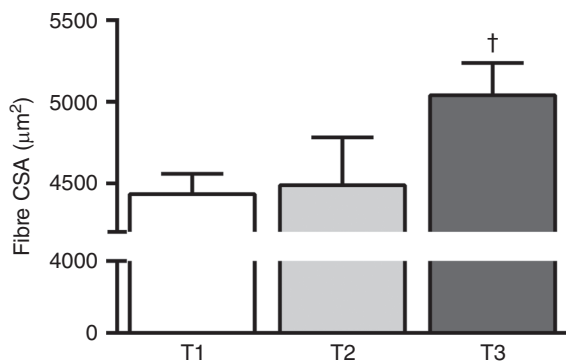

Figure 3. Fibre cross-sectional area (CSA) at the first week (T1), third week (T2) and tenth week (T3) of resistance training
†Significantly different ($P < 0.05$) from T1 and T2. Values are means (SEM).

Z-band streaming

Figure 6 shows the two scores of the magnitude of muscle damage through Z-band streaming. Figure 6*A* reveals the change after RE in the percentage of Z-band streaming area per total fibre area at T1, T2 and T3. The change from baseline in the areas with Z-band streaming after RE at T1 was greater than at T3 only ($P = 0.006$) (Fig. 6*A*). Figure 6*B* depicts the change in the percentage of fibres that presented any sign of Z-band streaming after RE at the three training phases (i.e. T1, T2 and T3). At T1, there was a higher percentage of affected fibres than at T2 and T3 ($P < 0.001$) (Fig. 6*B*).

Indirect markers of muscle damage

Baseline values for MVC at T1, T2 and T3 are reported in Damas *et al.* (2016*a*). We reported there that the RT protocol was effective to increase muscle strength, i.e. MVC pre-RE at T3 was greater than before the beginning of RT (baseline at T1) (Damas *et al.* 2016*a*). All indirect markers of muscle damage (MVC, SOR and CK) showed significant changes from baseline at T1: MVC decreased and SOR increased at 24 h and 48 h and CK increased at 48 h compared to baseline ($P < 0.001$ for all). At T2, only SOR slightly increased at 24 h *versus* baseline ($P = 0.033$), but no other change was found at T2 or T3 from baseline for the other variables. In addition, MVC and SOR at 24 h and 48 h T1, and CK at 48 h T1, were significant different ($P < 0.001$) from the same time points at T2 and T3. The CK value was slightly higher at 48 h T2 compared with 48 h T3 ($P = 0.035$) (Table 1).

Correlation analysis

No significant correlations were found between any hypertrophic marker (MyoPS, fCSA and VL CSA) and indirect
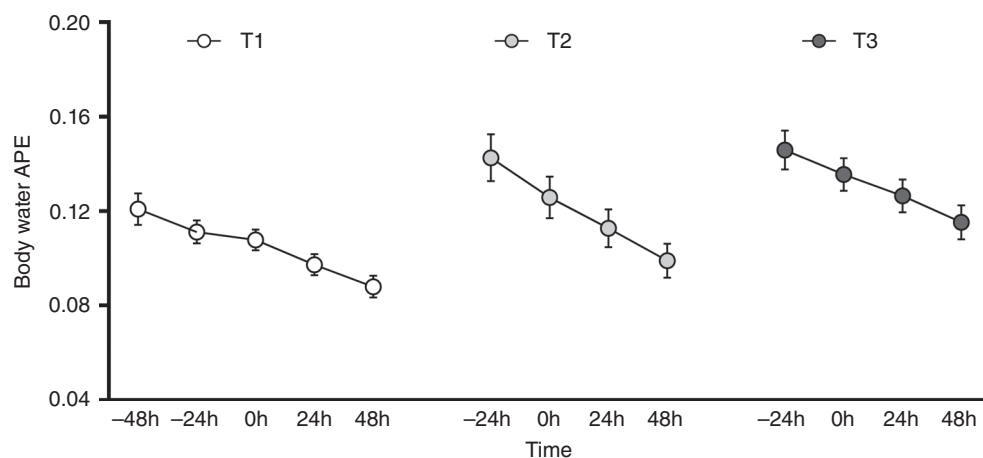

Figure 4. Time course of body water enrichment (atom% excess, APE) throughout the experimental period
T1: first week, T2: third week, T3: tenth week of resistance training. Values are means (SEM).

markers of muscle damage, i.e. MVC, SOR or CK at any time point. For the relationship between Z-band streaming and hypertrophic markers, as well as for correlations between acute and chronic hypertrophic outcomes, the main results are depicted in Table 2. Significant ($P < 0.04$; $r \approx 0.9$) correlations were found between the integrated MyoPS (0–48 h) post-RE at T3 and the increase in fCSA (T3 − T1), and between the integrated MyoPS (0–48 h) post-RE at T2 and T3 and the increase in VL CSA (T3 − T1) (VL CSA data from Damas *et al.* 2016*a*).

Discussion

We discovered that both MyoPS and muscle damage were highest after an initial bout of RE, but neither were correlated with RT-induced skeletal muscle/fibre hypertrophy, which is in agreement with our earlier work (Mitchell *et al.* 2014). Despite the lack of correlation between initial MyoPS and muscle hypertrophy, we observed that early (T2) and later (T3) rates of MyoPS, while attenuated compared to T1, were correlated with muscle hypertrophy. We also report that directly measured
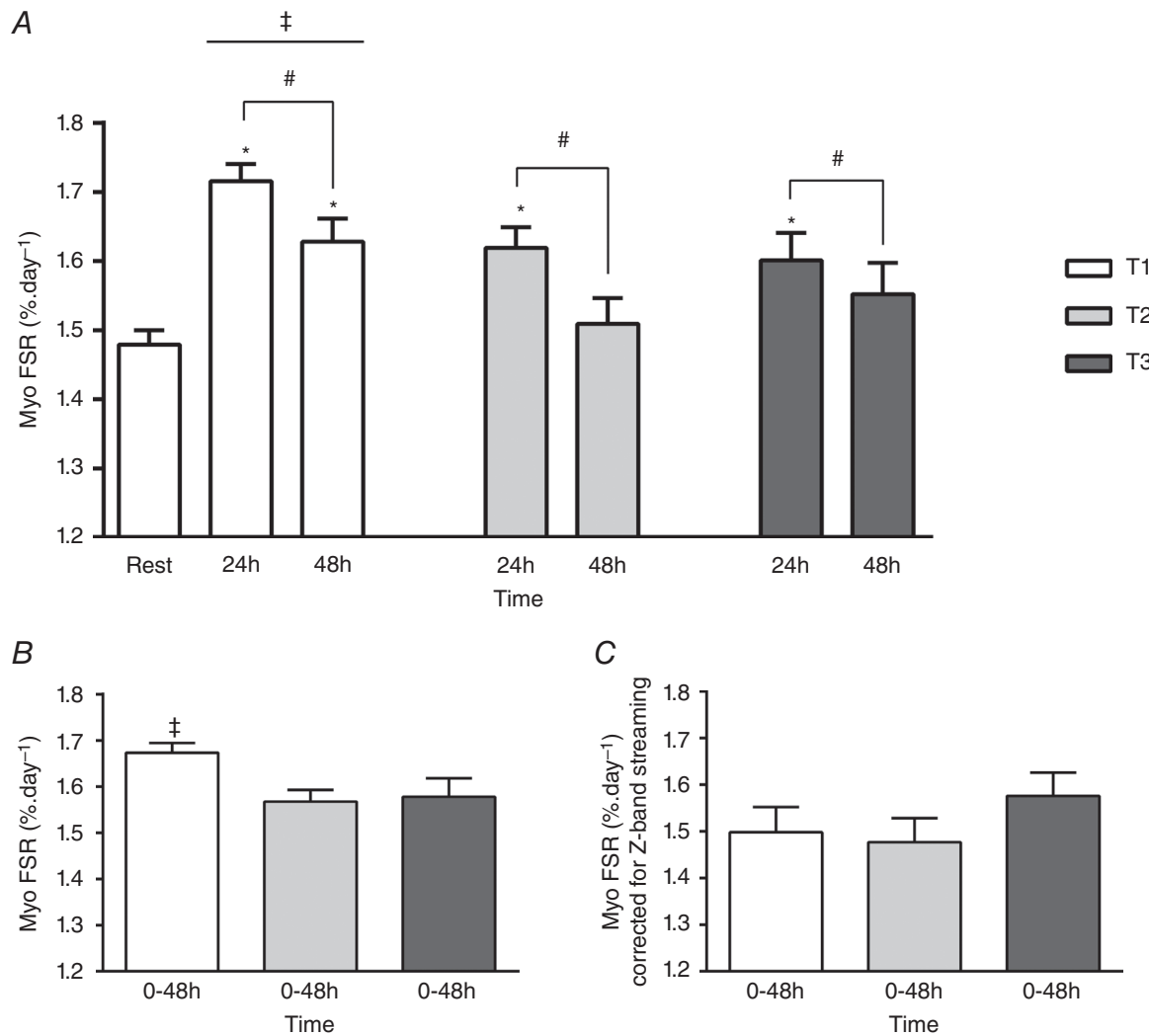

Figure 5. Myofibrillar (Myo) protein fractional synthetic rates expressed as: absolute (*A*), changes (*B*), and damage-corrected (*C*) rates
A, myofibrillar (Myo) fractional synthetic rate (FSR) at rest, and 24 h and 48 h following a single bout of resistance exercise at the first week (T1), third week (T2) and tenth week (T3) of resistance training. *Significantly different ($P < 0.05$) from rest at T1. #Main acute time effect (24 h significantly different ($P = 0.003$) from 48 h independent of training phase). ‡Main training phase effect (T1 significantly different ($P < 0.03$) from T2 and T3). *B*, integrated Myo FSR over the first 48 h after a single bout of resistance exercise at T1, T2 and T3. ‡Significantly different ($P < 0.05$) from T2 and T3. *C*, integrated Myo FSR over the first 48 h following a single bout of resistance exercise at T1, T2 and T3 normalized for the change (48 h − 0 h) in the amount (% of affected areas) of Z-band streaming (FSR × (100 − Z-band streaming)/100) at T1, T2 and T3, respectively. Values are means (SEM).

muscle damage, which was progressively mitigated throughout RT reaching a minimal magnitude at the end of 10 weeks of RT, did not correlate with MyoPS or hypertrophy at any time point during RT. Our data provide novel insight into the antecedents of RT-induced muscle hypertrophy showing that at both early and later training phases, when muscle damage progressively subsides, the change in MyoPS after RE is directed to support muscle fibre hypertrophy.

Our data are in agreement with the thesis that, in an untrained state, MyoPS would be increased to a larger extent than in a trained state (Damas *et al.* 2015). We used an integrated approach and measured MyoPS over days (as opposed to hours), with the participants in fed and fasted states, and showed that the rise in MyoPS is attenuated in a trained state (T3) in comparison with an untrained state (T1). Interestingly, we also show an early (at T2) attenuation in MyoPS post-RE compared with that observed at T1, with no difference between the attenuated MyoPS rates post-RE at T2 and T3. Despite a potent increase in integrated MyoPS in the first 48 h after the very first bout of RE, this rise did not correlate with long-term muscle hypertrophy (Table 2); a finding that is congruent with previous results using infusion trials (Mayhew *et al.* 2009; Mitchell *et al.* 2014) in which hour-to-hour protein synthetic rates were measured. Importantly, we demonstrate that the change in integrated 0–48 h MyoPS post-RE at the end of the RT period (T3) was correlated ($r = 0.91$) with the increase in fCSA after 10 weeks of RT, and further, the changes in MyoPS 0–48 h post-RE at both T2 and T3 were significantly and also highly correlated ($r = 0.86$ and $r = 0.94$) with the increases in VL muscle CSA (data from Damas *et al.* 2016*a*) after 10 weeks of RT (Table 2). It is worth noting that the integrated 0–48 h MyoPS response at T2 showed a reasonable agreement ($r \approx 0.7$) with the increase in

fCSA after 10 weeks of RT. A recent study measured the integrative MyoPS throughout the first 3 weeks of RT (participants trained three times a week) and found a significant correlation ($r \approx 0.7$) with the increase in muscle thickness (Brook *et al.* 2015). The authors also showed a down-regulation in MyoPS with further training (decline in 3 weeks integrated – weeks 3–6 – MyoPS), which returned to pre-training values. In agreement with Brook *et al.* (2015), our results demonstrate an early attenuation of MyoPS at T2 *versus* T1 with no further changes from T2 to T3; however, we observed significant increases in MyoPS during the first 24 h of post-RE at T2 and T3. While our results may appear discordant with those of Brook *et al.* (2015), we propose that the difference is explained by the fact that our measurements were over days (24 h and 48 h) while theirs were taken over weeks. Based on our present and previous data (Phillips *et al.* 1997), MyoPS peaks 24 h post-RE and begins to decline, thus measuring MyoPS across 3 weeks (Brook *et al.* 2015) would have yielded a less temporally resolved picture of the increase in MyoPS after each RE bout. Additionally, we show that beyond 3 weeks of RT the changes in MyoPS post-RE are attenuated compared to the change post-RE in the first week. Thus, what Brook *et al.* (2015) reported, we suggest, were progressively smaller increments in MyoPS after each RE beyond 3 weeks and these increments would have been 'diluted' by measuring MyoPS over a 3 week period. In turn, this would lead to a reduction in overall MyoPS returning to pre-training values and a lack of correlation between MyoPS with muscle hypertrophy in that phase of RT. Therefore, our results expand on those of Brook *et al.* (2015) by allowing a more temporally resolved picture of MyoPS during an RT programme. We report that integrative 48 h post-RE MyoPS is already lower at an early phase (week 3; T2) but is not further attenuated after 10 weeks of RT. Importantly, and in general agreement
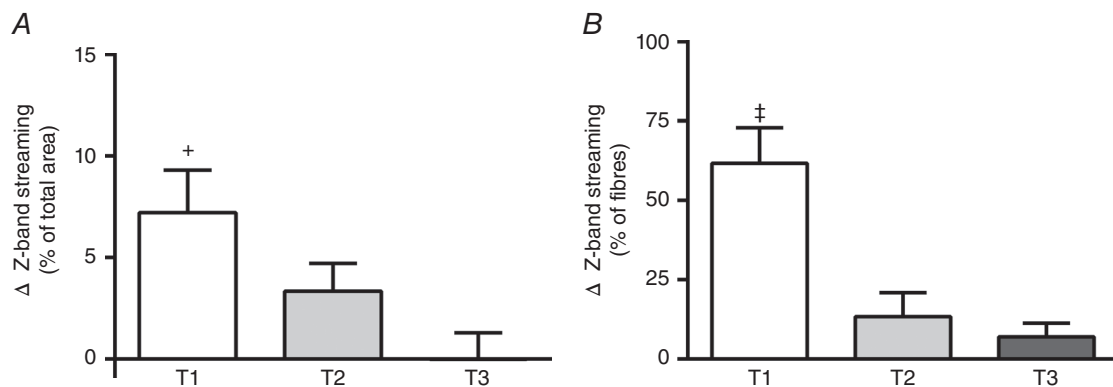

Figure 6. Muscle damage expressed as Z-line streaming
A, change from baseline in the percentage of Z-band streaming areas per total fibre area following a single bout of resistance exercise at the first week (T1), third week (T2) and tenth week (T3) of resistance training. *B*, change from baseline in the percentage of fibres that showed any sign of Z-band streaming following a single bout of resistance exercise at T1, T2 and T3. [+]Significantly different ($P < 0.05$) from T3. [‡]Significantly different ($P < 0.05$) from T2 and T3. Values are means (SEM).

Table 1. Changes in maximum voluntary contraction torque (MVC) from baseline (100%), absolute changes in muscle soreness (SOR) and absolute changes in creatine kinase (CK) activity level following a single bout of resistance exercise throughout resistance training

	T1			T2			T3		
	Baseline	24 h	48 h	Baseline	24 h	48 h	Baseline	24 h	48 h
MVC (% baseline)	100 (0)	77.8 (4.6)*‡	77.9 (5.2)*‡	100 (0)	96.7 (2.6)	98.2 (2.3)	100 (0)	94.4 (3.6)	97.7 (1.8)
SOR (mm)	0 (0)	40 (6)*‡	61 (8)*‡	0 (0)	10 (3)*	8 (2)	0 (0)	8 (2)	8 (3)
CK activity (U l^{-1})	119 (11)	—	416 (81)*‡	132 (24)	—	226 (33)$^{+}$	107 (11)	—	110 (9)

T1: first week of training, T2: third week of training, T3: tenth week of resistance training. *Significantly different ($P < 0.05$) from baseline within the training phase (T1, T2 and T3). ‡Significantly different ($P < 0.05$) from the corresponding time point at T2 and T3. $^{+}$Significantly different ($P < 0.05$) from the corresponding time point at T3. Values are means (SEM).

Table 2. Correlations between hypertrophic responses and muscle damage variables, and correlations between acute and chronic hypertrophic outcomes

Variable		Integrated MyoPS T1 (% day^{-1})	Integrated MyoPS T2 (% day^{-1})	Integrated MyoPS T3 (% day^{-1})	Increase in fCSA (%) (T3 − T1)	Increase in VL CSA (%) (T3 − T1)
Z-band streaming (48 h − baseline) T1 (% area)	r	0.558			−0.431	0.210
	P	0.094			0.393	0.560
Z-band streaming (48 h − baseline) T2 (% area)	r		−0.137		−0.534	−0.522
	P		0.705		0.275	0.122
Z-band streaming (48 h − baseline) T3 (% area)	r			−0.196	−0.077	−0.353
	P			0.613	0.884	0.317
Z-band streaming (overall) (% area)	r				−0.432	−0.056
	P				0.393	0.878
Increase in fCSA (%) (T3 − T1)	r	−0.602	0.687	0.911[a]		
	P	0.206	0.131	0.032*		
Increase in VL CSA (%) (T3 − T1)	r	−0.221	0.864[a]	0.946[a]		
	P	0.540	0.001*	0.000*		

CSA: cross-sectional area; fCSA: fibre cross-sectional area; Integrated MyoPS: myofibrillar protein synthesis considering the first 48 h (i.e. 0–48 h) after a single resistance exercise bout; Z-band streaming (overall): calculation that took into account the total amount of area with Z-band streaming divided by total area analysed at T1, T2 and T3 altogether; VL: vastus lateralis; T1: first week, T2: third week, T3: last week of training. *Significant ($P < 0.05$) correlation. [a]High correlation between variables. VL CSA data from Damas *et al.* (2016*a*).

with Brook *et al.* (2015), we report that MyoPS at T2 was correlated with muscle hypertrophy (increase in VL CSA at T3 − T1), but we expand the findings showing that the change in MyoPS post-RE after 10 weeks of RT (at T3) is also highly correlated with hypertrophy (increase in fCSA and VL CSA at T3 − T1).

Our RT programme promoted significant muscle fibre hypertrophy (∼14%) that was evident only at the end of the RT period (T3; Fig. 3). This finding corroborates our previous proposal that early increases in VL muscle CSA measured using ultrasound imaging are, to some extent, due to oedematous muscle swelling (i.e. not true fibre hypertrophy) probably induced by muscle damage caused by the early RE bouts in an RT programme (Damas *et al.* 2016*a*,*b*). As expected, the first RE bout promoted significant muscle damage, as evidenced by both direct (Z-band streaming) and indirect (MVC, SOR and CK) measurements. The magnitude of Z-band streaming

found at T1 is lower than in previous investigations utilizing eccentrically biased RE (Gibala *et al.* 1995; Beaton *et al.* 2002*a*). Accordingly, the magnitude of changes in indirect markers are representative of mild-to-moderate damage (Paulsen *et al.* 2012) and are not indicative of *loss* of myofibrillar proteins (Stupka *et al.* 2001; Yu & Thornell, 2002; Crameri *et al.* 2004, 2007; Corona *et al.* 2010; Murphy *et al.* 2013). Indeed, all changes in indirect damage markers returned to baseline values at T2 and the magnitude of change in MVC, SOR and CK after RE at T2 was substantially attenuated. This RT-induced attenuation of damage Z-band streaming was not complete at T2 (Fig. 6). The early *potent* repeated bout effect (i.e. within the first RE bouts) extensively demonstrated in the literature (Nosaka *et al.* 2007; Chen *et al.* 2009, 2012, 2013; Muthalib *et al.* 2011; Starbuck & Eston, 2012) is only manifest by indirect markers (e.g. MVC, SOR and CK), not by Z-band streaming, at least when using a continuous traditional RT regimen. After 19 RE bouts (at T3) we were able to detect an attenuation of Z-band streaming, but we acknowledge that the attenuation might have happened earlier (somewhere between T2 and T3). Previously, a study using a cross-sectional design demonstrated that the amount of muscle damage shown by Z-band streaming after RE is higher for untrained *versus* trained individuals (Gibala *et al.* 2000). We expand these findings providing longitudinal (intra-individual) evidence of the difference in Z-band streaming at different training status.

We noted a moderate correlation between the magnitude of Z-band streaming and the change in MyoPS post-RE at T1 ($r \approx 0.6$, $P = 0.09$; Table 2). This, added to the fact that MyoPS at T1 does not correlate with hypertrophy, leads us to speculate that the increase in MyoPS seen at T1 is related more to 'repair' of damaged proteins resulting from the unaccustomed RE bout. Our proposal is that the initial response of MyoPS may be directed more toward increasing sarcomeres in series (Yu *et al.* 2004), rather than in parallel (i.e. muscle hypertrophy). Indeed, Fig. 5*C* supports this notion since when the overall change in MyoPS in the 48 h after RE is normalized to the percentage area with Z-band streaming (which can be considered a rough way of accounting for the amount of MyoPS required to repair a 'damaged' area), the increase in MyoPS at T1 (Fig. 5*A* and *B*) is no longer significant and all three training phases (T1, T2 and T3) showed similar MyoPS values. Thus, when the stress of RE is novel, it promotes a potent increase in MyoPS to support muscle repair rather than promote increases in muscle fibre CSA. Nonetheless, the MyoPS response rapidly adapts and by the third week of RT (T2) or even earlier, indirect markers of muscle damage show potent attenuation post-RE, and even though Z-band streaming is still present (albeit attenuated in magnitude), MyoPS is directed more to support an increase in fCSA.

Altogether, our data indicate that muscle damage does not have a role in skeletal muscle hypertrophic responses during prolonged RT. That is, individuals who present more muscle damage do not necessarily have a greater hypertrophic response.

One limitation of our work was that we did not measure resting MyoPS at T2 and T3. A variety of resistance training studies have shown either no change (Phillips *et al.* 1999; Tang *et al.* 2008) or increased (Phillips *et al.* 2002; Kim *et al.* 2005) hourly rates of mixed but not myofibrillar protein synthesis (Kim *et al.* 2005; Wilkinson *et al.* 2008). Thus, we speculate that either a small increase or, more likely, no change would have occurred in resting MyoPS rates at T2 and T3, compared to T1. Importantly, however, despite our choice not to measure resting MyoPS at T2 and T3 we still observed that the change in MyoPS after RE was lower at T2 and T3 than at T1 and that there were significant correlations between the integrated MyoPS response (i.e. 0–48 h MyoPS after RE at T2 and T3) and hypertrophy. We speculate that our ability to see these correlations was due to the fact that changes in resting MyoPS with RT are not the main reason for hypertrophy and that despite an attenuated change in MyoPS after RE as RT progresses the periodic increases in MyoPS are still the main contributor to protein accrual.

In summary, we report that the first RE bout in an RT programme promotes the largest change in MyoPS, marked Z-band streaming, and changes in indirect markers of damage, but none of these variables correlate with hypertrophy. Early in an RT programme (week 3, T2) there was an attenuation of MyoPS after RE, Z-band streaming was present, but attenuated. Also, MyoPS, but not muscle damage, correlated with hypertrophy at week 3. At 10 weeks, changes in damage markers (including Z-band streaming) after RE were potently attenuated, but changes in MyoPS after RE were similar to those observed at the third week of RT and still highly correlated with muscle/fibre hypertrophy. When changes in MyoPS were normalized to the area of Z-band streaming, the response of MyoPS was similar at all times. We propose that the initial RE bout(s) promotes a robust but potentially non-hypertrophy-directed but instead 'repair-oriented' increase in MyoPS; nonetheless, early on in the RT programme, adaptations in the changes in MyoPS after RE occur to promote primarily muscle hypertrophy. Thus, muscle hypertrophy is the result of accumulated increases in MyoPS after RE mainly after attenuation of muscle damage.

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Additional information

Competing interests

None declared.

Author contributions

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