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Costamere remodeling with muscle loading and unloading in healthy young men

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Abstract

Costameres are mechano-sensory sites of focal adhesion in the sarcolemma that provide a structural anchor for myofibrils. Their turnover is regulated by integrin-associated focal adhesion kinase (FAK). We hypothesized that changes in content of costamere components (beta 1 integrin, FAK, meta-vinculin, gamma-vinculin) with increased and reduced loading of human anti-gravity muscle would: (i) relate to changes in muscle size and molecular parameters of muscle size regulation [p70S6K, myosin heavy chain (MHC)1 and MHCIIA]; (ii) correspond to adjustments in activity and expression of FAK, and its negative regulator, FRNK; and (iii) reflect the temporal response to reduced and increased loading. Unloading induced a progressive decline in thickness of human vastus lateralis muscle after 8 and 34 days of bedrest (−4% and −14%, respectively; $n = 9$), contrasting the increase in muscle thickness after 10 and 27 days of resistance training (+5% and +13%; $n = 6$). Changes in muscle thickness were correlated with changes in cross-sectional area of type I muscle fibers ($r = 0.66$) and beta 1 integrin content ($r = 0.76$) at the mid-point of altered loading. Changes in meta-vinculin and FAK-pY397 content were correlated ($r = 0.85$) and differed, together with the changes of beta 1 integrin, MHCI, MHCII and p70S6K, between the mid- and end-point of resistance training. By contrast, costamere protein level changes did not differ between time points of bedrest. The findings emphasize the role of FAK-regulated costamere turnover in the load-dependent addition and removal of myofibrils, and argue for two phases of muscle remodeling with resistance training, which do not manifest at the macroscopic level.

Key words: atrophy; focal adhesion kinase; human; hypertrophy; load; muscle; myofibril; vinculin.

Introduction

Skeletal muscle size shows a pronounced mechano-dependence, notably increasing in size with chronic overloading and decreasing in size with chronic unloading, as for example during experimental bedrest (Loughna et al. 1986). Changes in mean muscle cross-sectional area (CSA) and fascicle pennation angle can be observed within a few days of altered loading in humans (Narici & Maganaris, 2007; Hackney & Ploutz-Snyder, 2012). For instance, atrophy is detectable as early as after 7 days of bedrest (Ferrando et al. 1995) in anti-gravity muscle m. vastus lateralis, and aggravates progressively with prolonged unloading until 6 months (Bloomfield, 1997; Hackney & Ploutz-Snyder, 2012). Similarly hypertrophy of m. vastus lateralis can be detected after as few as 10 sessions of eccentric-type resistance exercise in 3 weeks, further increasing with the progression of the training period (Seynnes et al. 2007).

Changes in muscle size are largely driven by modified content of myofibrils, which involves adaptations of both slow- and fast-type fibers (Fry, 2004; Borina et al. 2010). With unloading, atrophy is observed for both slow type I, and fast type IIA and IIX muscle fibers (Bloomfield, 1997; Fitts et al. 2001; Hackney & Ploutz-Snyder, 2012). Based on changes in myosin heavy chain (MHC) expression with prolonged unloading, there may also be a conversion of slow-

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into fast-type muscle fibers, leading to a relative increase in fast- over slow-type MHC content (Bloomfield, 1997; Hackney & Ploutz-Snyder, 2012). An increase in nitrogen excretion suggests that altered myofibrillar makeup contributes to the observed muscle atrophy after 7 days of unloading in humans (Bloomfield, 1997). More recently, the use of stable isotopes has enabled to show a 50% reduction in myofibrillar protein synthesis within 10 days of lower limb unloading in humans (de Boer et al. 2007a,b). Conversely, an increase in myofibrillar synthesis rate implicates the contribution of increased MHC synthesis to the elevated CSA of both slow- and fast-type muscle fibers after resistance-type exercise (Williamson et al. 2001; Fry, 2004; Wilkinson et al. 2008). Despite its apparent importance (Adams et al. 2003; Chopard et al. 2009), the question arises of how such myofibrillar alterations are integrated between the molecular and the architectural level in muscle fibers that undergo adaptive changes.

Sites of focal adhesion in the sarcolemma (costameres) are critical for the attachment of myofibrils to the fiber periphery (Ervasti, 2003; Grounds et al. 2005). They assemble through the binding of cytoskeletal and signaling molecules to the intracellular inside of integrin-type and/or dystrophin/sarcoglycan-type extracellular matrix receptors (Miyamoto et al. 1995; Ervasti, 2003; Grounds et al. 2005; Trimarchi et al. 2006). Thereby costameres provide an anchor for the intermediate filaments that hold sarcomeres in register and establish a physical link between neighboring muscle fibers via the interstitium (Pardo et al. 1983; Ervasti, 2003; Grounds et al. 2005; Ramaswamy et al. 2011). Assembly of new costameres is essential for myofibrillogenesis in culture by providing a platform for the attachment of myofibril/myosins (Quach & Rando, 2006). The association between costameres and myofibrils is of great interest given that mechanical factors exert important control over the expression of costamere components and fiber size in anti-gravity muscle (Fluck et al. 1999; Gordon et al. 2001; Chopard et al. 2002, 2005; Anastasi et al. 2008). For example, expression of the costamere components gamma- and meta-vinculin, talin and b1-integrin is compromised with prolonged muscle unloading and muscle inactivity when muscle sarcomeres are lost (Chopard et al. 2002, 2005; Anastasi et al. 2008). By contrast, concomitant resistance-type exercise prevents alterations in costamere components in unloaded muscle (Chopard et al. 2005). Interestingly, costamere component expression also varies between fiber types (Shear & Bloch, 1985; Bozyczko et al. 1989; Schröder et al. 1997; Williams et al. 2000; Fluck et al. 2002; Thoss et al. 2013). Owing to their role in myofibril attachment, the mechano-regulation of costamere components is suggested to reflect active phases of muscle remodeling and fiber-type transformation with altered loading.

The integrin-associated focal adhesion kinase (FAK) is instrumental for costamerogenesis in culture (Quach & Rando, 2006). Overexpression of FRNK, which competes with FAK for binding to focal adhesions, implies that this involves the control over focal adhesion turnover via the interaction between focal adhesion components (Ilic et al. 1995; Schlaepfer et al. 2004). Phosphorylation of FAK at tyrosine residue 397 is an important control point of this regulation. It alters the capacity of FAK to interact with binding partners such as the tyrosine kinase c-src, enabling FAK to develop its full catalytic activity, phosphorylate auxiliary sites and enter interactions with further binding partners (Schlaepfer et al. 1999) that mediate the clustering of cytoskeletal and signaling proteins to integrins (Miyamoto et al. 1995). A number of observations highlight that both phosphorylation of Y397 and auxiliary sites, as well as the content of FAK, is load-regulated (Li et al. 1997; Aikawa et al. 2002; Torsoni et al. 2003; Lal et al. 2007). For instance, FAK-pY397 content is increased in relation to enhanced FAK protein content after the first day of chronic overload in rat soleus muscle (Fluck et al. 1999; Gordon et al. 2001; Durieux et al. 2009). Conversely, muscle unloading downregulates FAK tyrosine phosphorylation within 7 days in rat and human skeletal muscle, and this is confounded by an upregulation of FAK protein content in rat muscle (Gordon et al. 2001; de Boer et al. 2007b). Likewise, components of the dystrophyin/sarcoglycan-type receptor demonstrate mechano-regulated expression and are involved in mechanical signal transduction in skeletal muscle (Barton, 2006; Chopard et al. 2005). Increased FAK amount in rodent skeletal muscle appears also to promote the load-regulated activation of the serine/threonine kinase p70S6K (the positive regulator of protein synthesis), which is robustly associated with increased protein synthesis and hypertrophy with muscle overload (Baar & Esser, 1999; Terzis et al. 2008; Wilkinson et al. 2008; Klossner et al. 2009; Carter & Flueck, 2012).

We reasoned that loading-dependent plasticity of muscle size is integrated through costamere turnover via a load-regulated process that involves level alterations of FAK and FAK-pY397 concentration. The functional implication of FAK in the hypothesized mechano-regulation of costamere remodeling was studied by quantifying the concentration of the focal adhesion components, meta- and gamma-vinculin, beta 1 integrin and p70S6K in relation to concentration changes in FAK, FAK-pY397 and its inhibitor FRNK during increased loading and unloading of human vastus lateralis muscle. We thus hypothesized that the changes in costamere components through the course of increased muscle loading with eccentric-type resistance training and unloading by bedrest would be inter-related with FAK-pY397. As costameres are connected to the cytoskeletal elements that hold sarcomeres in register, we expected that costamere protein levels exist in a constant proportion to muscle size (muscle thickness or CSA), except during phases of muscle remodeling characterized by the formation or removal of myofibrils (i.e. fiber transformation and atrophy). This was tested by comparing the changes in muscle

size with those of costamere protein levels during the course of altered loading of m. vastus lateralis in humans. Time points for the sampling of bioptic material were selected on the basis of documented anatomical changes with the two loading interventions (Ferrando et al. 1995; de Boer et al. 2007a; Seynnes et al. 2007).

Materials and methods

Experimental design

Anti-gravitational muscle (m. vastus lateralis) of untrained human subjects was subjected either to unloading by bedrest (experimental Group 1) or to overloading by resistance training (experimental Group 2). In both groups, anatomical measures were taken to estimate muscle size and muscle fiber CSA, and a muscle sample was collected to determine the content of costameric proteins (FAK, FRNK, beta 1 integrin, meta-vinculin, gamma-vinculin) and the phosphorylation status of FAK at pY397, and where appropriate the content of p70S6K. All subjects gave their written informed consent to participation in the study, which had been approved by the Ethical Committee of the University of Primorska for subjects of Group 1 and by the local Ethics Committee of Manchester Metropolitan University for subjects of Group 2. Muscle tissue was collected and stored under the United Kingdom's Human Tissue Act.

Bedrest (Group 1)

These experiments were carried out in the Orthopedska Bolnica Hospital in Valdoltra, Ankaran (Slovenia) under medical supervision. Nine healthy Caucasian young men (age: 24.3 ± 2.6 years; stature: 179.7 ± 8.0 cm; body mass: 76.4 ± 10.4 kg) from the Slovenia area were recruited for this study via advertisement in a student job portal and via word of mouth. Muscle thickness was determined after the 8th and 34th day of bedrest (see 'Muscle anatomy' below). On this occasion a muscle biopsy sample was collected with a conchotome under local anesthesia from the belly portion of the vastus lateralis muscle (~80–150 mg). Samples were snap-frozen in liquid nitrogen and stored until use at −80 °C.

Resistance training (Group 2)

Six healthy Caucasian men (age: 22.3 ± 1.5 years; stature: 175.2 ± 3.4 cm; body mass 71.0 ± 4.6 kg) from the student population of Manchester Metropolitan University participated in this study. Unilateral leg extension was performed three times per week for 9 weeks on a flywheel ergometer (YoYo Technology, Sweden; Seynnes et al. 2007). In brief, resistance is produced from the inertia of the flywheel during both concentric and eccentric contraction phases, by virtue of the unwinding/rewinding of a strap connecting the flywheel to the lever arm. Each session consisted of one set of seven submaximal leg extensions as a warm-up, and was proceeded by four sets of 10 maximal coupled concentric and eccentric contractions, with 2-min rest periods between sets. Subjects were tested for vastus lateralis muscle thickness and quadriceps femoris CSA on three occasions (see 'Muscle anatomy' below): at baseline, 4 weeks before the first training session (pre-training); after the 10th training session (mid-training); and after the 27th training session (post-training). Bioptic samples were collected pre-training, mid- and post-training from lignocaine-anesthetized vastus lateralis muscle. For the training samples the interval between the preceding exercise bout and muscle sampling was 4–6 h.

Muscle anatomy – Group 1

Muscle thickness was measured by using B-mode ultrasound scanning (Mylab 25, 13–4 MHz, linear array transducer probe LA523; Esaote Biomedica, Geneva, Italy). Scans were performed at mid-muscle length along the mid-sagittal axis of the vastus lateralis, perpendicular to the lower aponeurosis plane. Thickness was defined as the average of three equidistant measurements along the scan width, between the superficial and deep aponeuroses of the muscle. The consistency of ultrasound scanning between the testing sessions was ensured by recording the location and orientation of the transducer on transparent acetate paper.

Group 2

The methods for assessing maximum anatomical CSA of the vastus lateralis muscle have been described in detail elsewhere (Erskine et al. 2009). Briefly, the upper leg was scanned using a 0.2-T magnetic resonance imaging scanner (G-Scan, Esaote Biomedica, Genoa, Italy), adhering to a Turbo 3D T1-weighted sequence with the following scanning parameters: time of repetition 40 ms; time to echo 16 ms; matrix 256×256; field of view $180\ mm \times 180\ mm$; slice thickness 2.8 mm; interslice gap 0 mm. Contiguous axial slices were taken from the tibiofemoral joint to the iliac crest perpendicular to the femur, with the participant in the supine position. From the contiguous slices, the maximal anatomical CSA of the vastus lateralis muscle was identified and manually outlined (Osirix 2.7.5, Osirix Foundation, Geneva, Switzerland). Muscle thickness was quantified by ultrasound with the same approach used for Group 1.

CSA of muscle fibers

Twelve-micrometer cryosections were prepared from muscle biopsies and subjected to fiber typing using mouse anti-type II MHC and Alexa Fluor 555-coupled secondary anti-mouse antibody (DAKO) essentially as described (Fluck et al. 1999; Gordon et al. 2001; Durieux et al. 2009; Klossner et al. 2009). The fluorescent signal was digitally recorded from different microscopic fields with standardized settings using a TCS SP5 confocal microscope ($10 \times$ objective and $4 \times$ zoom; Leica Microsystem CMS, Milton Keynes, UK). Image files were exported and the CSA of stained (type II) and non-stained (type I) fibers in a given microscopic field was quantified using image J 1.6.0_33 J (http://imagej.nih.gov/ij). Numerical values for the same fiber type from the different microscopic fields of a muscle cross-section were pooled to calculate for each muscle biopsy the mean CSA of type II and type I fibers, and the percentage in muscle area covered by type I fibers (Flueck et al. 2011). On average, 45 and 84 type I and type II muscle fibers, respectively, were counted per muscle biopsy (subject). The mean coefficient of variance for the estimation of CSA per subject was 4%.

Immunodetection of FAK in sections

Staining of muscle sections for FAK was carried out with fluorescence essentially as described in Klossner et al. (2009).

Twelve-micrometer cryosections were freshly prepared from pre- and post-unloading samples of subjects and assembled onto the same slides. Sections were left to dry and fixed with 4% paraformaldehyde/0.1% Triton X-100 for 30 min, quenched with 3% H_2O_2 and blocked in 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Subsequently, sections were incubated with a 1 : 100 dilution of FAK C-terminal antibody (Fluck et al. 1999; Gordon et al. 2001; Chopard et al. 2002, 2005; Anastasi et al. 2008) and mouse anti-type I MHC (Durieux et al. 2009) in 3% BSA–PBS overnight at 4 °C, followed by washes in PBS and incubation with a 1 : 400 dilution of Alexa Fluor 488-coupled secondary anti-rabbit antibody (DAKO) and Alexa Fluor 555-coupled secondary anti-mouse antibody (DAKO). Nuclei were stained using 5-min incubations with TO-PRO3 (Invitrogen).

Fluorescence and digital phase contrasts were recorded at standardized settings (10 × objective and 4 × zoom) using a TCS SP5 confocal microscope (Leica Microsystem CMS, Milton Keynes, UK).

Subsequently, image files were taken from different microscopic fields of a stained muscle section and exported as tiff files. The signal intensity of FAK at the sarcolemma was inspected and quantified using image J 1.6.0_33 J (http://imagej.nih.gov/ij). Fibers were categorized for fiber type and sarcolemmal FAK content (positive or negative). Fibers were classified as positive when staining was identified along at least two edges of the generally squared human muscle fibers, as described elsewhere (Fluck et al. 2002; Evans et al. 2008). The number of fibers in each of the four categories (i.e. type I-FAK+, type I-FAK−; type II-FAK+, type II-FAK−) was determined for each muscle from counting of different, non-overlapping microscopic fields. Statistical significance of changes in the frequency of FAK staining with unloading was assessed essentially as previously described (Fluck et al. 2003). First, the frequency of FAK staining at the sarcolemma of all combined muscle fibers of a given biopsy was assessed for a difference pre- vs. post-unloading with a Chi^2-test ($P < 0.05$). If statistical significance was reached, the percentage of FAK staining at the sarcolemma was calculated for type I and type II muscle fibers, respectively, and each muscle section, and compared for significance of differences post- vs. pre-unloading with a two-tailed paired *t*-test. On average, 23 and 59 type I and type II muscle fibers, respectively, were counted per muscle biopsy and subject.

Protein detection

Muscle was cryosectioned at 12 μm, and total homogenate was prepared in modified RIPA buffer (1% NP-40, 0.25% deoxycholate, 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, 1 μg mL^{-1} leupeptin, 2 μg mL^{-1} pepstatin, 1 μg mL^{-1} aprotinin; all reagents were received from Sigma). Protein concentration was quantified with bicinchoninic acid assay reagents against BSA standard (Pierce). Twenty micrograms of protein was separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, Western blotted onto nitrocellulose (Schleicher & Schuell). Gel loading was in a paired design with pre-, mid- and post-intervention samples of three subjects being applied in adjacent lanes per gel. The membrane was stained with Ponceau S to verify equal loading from the intensity of detected protein bands, including the major signal at 40 kDa that corresponds to skeletal alpha actin. Subsequently membranes were subjected to immunodetection with specific antibodies against slow-type MHC, fast-type MHC, FAK, FRNK, beta-1 integrin, gamma-vinculin, meta-vinculin, p70S6K, essentially as described (Fluck et al. 1999; Gordon et al. 2001; Durieux et al. 2009; Klossner et al. 2009). For the detection of fast-type myosin, the immunoblot was stripped after signal development for slow-type myosin by incubating for 30 min at 65 °C in 62.5 mM Tris, pH 6.8, 1% SDS, 0.7% beta-mercaptoethanol) with occasional shaking followed by extensive rinses in 0.5% Tween-20 in Tris-buffered saline. Content of FAK-pY397 was assessed in FAK-immunoprecipitates from the supernatant of 1 mg total protein as described (Klossner et al. 2009). Signal detection was carried out with enhanced chemoluminescence using a Geldoc system that was operated using Quantity One 1-D analysis software 4.6.1 (Bio-Rad Laboratories, Hemel Hempstead, UK). Signal intensity of the protein bands was determined using the rectangle density mode and background from an empty sample lane of equal size was subtracted. Background-corrected data were normalized to the mean values of the pre-samples for the respective gel; the values therefore reflect relative expression levels per total muscle protein.

Statistics

Alterations in 'muscle parameters' (muscle thickness, CSA, FAK, FRNK, FAK-pY397, meta-vinculin, gamma-vinculin, p70S6K, MHCI, MHCIIA) through the 'time course' (pre-, mid-, post-) of the particular 'loading condition' (i.e. unloading and overloading) were assessed with a repeated ANOVA for the repeated factor 'time course' using Statistica 9 (Statsoft, Tulsa, USA). A Wilcoxon test was used to localize the effect between time points of muscle unloading/overloading. Effects were called significant at $P < 0.05$. Pearson correlations were calculated to identify linear relationships that were deemed biologically significant if $r > 0.65$ and $P < 0.10$.

Results

Changes in muscle thickness and CSA

Table 1a and b, respectively, show the changes in muscle thickness with unloading by bedrest (Group 1) and overloading with eccentric-type of resistance training (Group 2). Muscle unloading produced a progressive reduction in thickness of vastus lateralis muscle (Table 1a). This was reflected by atrophy of type I muscle fibers (Table 2a). A tendency ($P = 0.08$ and 0.06 for changes mid- and post-bedrest, respectively) for a reduction in mean CSA of type II fibers was also observed (Table 2a).

Muscle overload increased the thickness and maximal anatomical CSA of vastus lateralis muscle (Table 1b). Neither the daily nor sessional percentage change in muscle thickness differed between the first and second intervention phase, either for bedrest ($P = 0.58$) or resistance training ($P = 0.72$). Mean CSA of type I (+34%), but not type II, muscle fibers was increased with resistance training, and this differed between the first and second phase of training (Table 2b). The percentage of type I fibers was 40% and 41%, respectively, before bedrest and resistance training, and this was not affected by the intervention ($P > 0.19$). Changes in CSA of type I and type II muscle fibers over all

Table 1 Opposite changes in muscle size with increased and reduced muscle loading. (a) Median (minima, maxima) and SE of muscle thickness (in mm) and percentage changes (mid- vs. pre-; post- vs. mid-) in vastus lateralis after 8 days (mid) and 34 days (post) of muscle unloading by bedrest ($n = 9$). (b) Median and SE of muscle thickness, maximal anatomical CSA and its percentage changes in vastus lateralis after 10 (mid) and 27 (post) sessions of overloading by resistance-type training ($n = 6$).

(a) Bedrest

	Pre-		Mid-		Post-
Thickness (mm)‡	22.1 (14.3, 28.6) ± 1.8		21.6 (13.2, 27.7) ± 1.9*		19.0 (11.0, 26.1) ± 1.6*
Delta (%)		−2.7 ± 0.9		−16.8 ± 2.4†	
Delta (% per session)		−0.34 ± 0.11		−0.49 ± 0.07	

(b) Resistance training

	Pre-		Mid-		Post-
Thickness (mm)‡	23.1 (17.0, 26.2) ± 1.6		24.7 (18.2, 26.3) ± 1.5		24.9 (19.4, 29.4) ± 1.6*
Delta (%)		3.7 ± 2.2		13.6 ± 2.7	
Delta (% per session)		0.37 ± 0.22		0.51 ± 0.10	
Maximal CSA	27.7 (18.5, 35.3) ± 2.4		27.3 (20.9, 35.1) ± 2.2		30.2 (21.9, 38.6) ± 2.6*
Delta (%)		4.8 ± 2.6		5.5 ± 2.1	

*,†$P < 0.05$ vs. pre-intervention levels and the 'mid- vs. pre-' comparison (Wilcoxon test).
‡Significant effect of 'time course' (repeated ANOVA).
CSA, cross-sectional area.

Table 2 Fiber CSA with altered muscle loading. (a, b) Median and SE of changes in mean CSA of type I and type II muscle fibers in vastus lateralis and after 8 days (mid-) and 34 days (post-) of bedrest (a; $n = 4$); and mid- (10 sessions) and post- (27 sessions) resistance-type training (b; $n = 5$).

(a) Bedrest

	Pre-		Mid-		Post-
CSA (μm^2)					
Type I	6113 ± 734		4515 ± 633*		4244 ± 884*
Delta (%)		−27.1 ± 2.0		−29.5 ± 8.7	
Type II	7245 ± 1256		5898 ± 1049†		5663 ± 1397†
Delta (%)		−10.0 ± 7.9		−14.8 ± 7.5	

(b) Resistance training

	Pre-		Mid-		Post-
CSA (μm^2)					
Type I‡	4530 ± 1151		4690 ± 1200†		6058 ± 1095*
Delta (%)		8.5 ± 2.5		16.1 ± 13.1†	
Type II	6135 ± 1337		6353 ± 798		6863 ± 744
Delta (%)		3.6 ± 8.7		11.9 ± 11.1†	

*,†$P < 0.05$ and $0.05 \leq P < 0.10$ vs. pre-intervention levels (Wilcoxon test).
‡Significant effect of 'time course' (repeated ANOVA).
CSA, cross-sectional area.

interventions were correlated with those of muscle thickness (Fig. 5A). Mid-way into the combined response to unloading or overloading, only the changes in CSA of type I fibers were correlated with those of muscle thickness (Fig. 5B).

Molecular factors of muscle size regulation during bedrest and resistance training

Molecular parameters of muscle size regulation (p70S6K, MHCI, MHCIIA) demonstrated an interaction effect between

the 'time point' × 'loading condition' ($P = 0.04$). Bedrest did not modify the content of MHCI or MHCII, or p70S6K (Fig. 1a,b,e). The content of the slow MHC, MHC I, increased at the mid-point of resistance training, and p70S6K protein was reduced in the end- vs. the mid-point of training (Fig. 1c,d,f).

Costameric protein expression with altered muscle loading

There was an interaction effect between the 'time point' × 'loading condition' for the two parameters associated with costameres, FAK-pY397 and meta-vinculin. Levels of both parameters were not altered in response to bedrest (Fig. 2). With muscle overload a biphasic response of both FAK-pY397 and meta-vinculin, and gamma-vinculin content was evident; all increasing between the mid- and end-point of resistance training (Fig. 3b,c,e,f). Changes in meta-vinculin and FAK-pY397 content were correlated over all sampled data points ($r = 0.80$), and this was pronounced when only changes at the end of the intervention were compared ($r = 0.85$). Muscle overload also increased beta 1 integrin mid- but not at the end of resistance training (Fig. 3d,f).

Levels of FAK and its inhibitor, FRNK, per total protein were not altered by muscle overload, but the content of both was reduced at the mid- and end-point of the unloading protocol (Fig. 2a,e). Microscopic examination visualized staining of FAK at the sarcolemma and locations in the sarcoplasma (Fig. 4). Sarcolemmal FAK immunoreactivity was significantly reduced after bedrest in type II muscle fibers (Fig. 4d).

Associations between muscular changes with altered loading

A number of linear relationships were identified between alterations in costamere protein content and muscle structure in the mid-point of atrophy and hypertrophy (Fig. 5B). This concerned correlations between percentage changes in beta 1 integrin content and muscle thickness ($r = 0.76$). Similarly, changes in FAK-pY397, meta-vinculin and b1 integrin content were correlated to changes in CSA of type I fibers after resistance training ($r > 0.65$). The changes in percentage of muscle area covered by type I fibers were positively correlated to changes in the costamere components gamma-vinculin, b1 integrin and type IIA MHC mid-way into altered loading, i.e. $r = 0.66$, 0.65 and 0.96, respectively. Conversely, changes in FAK-pY397 content mid-way into altered loading were negatively correlated to those of MHCI content ($r = -0.67$).

Discussion

Skeletal muscle mass is highly sensitive to alterations in mechanical loading (Flueck & Goldspink, 2010). A number of signaling factors/pathways, i.e. IGF-AKT-mTOR-p70S6K,

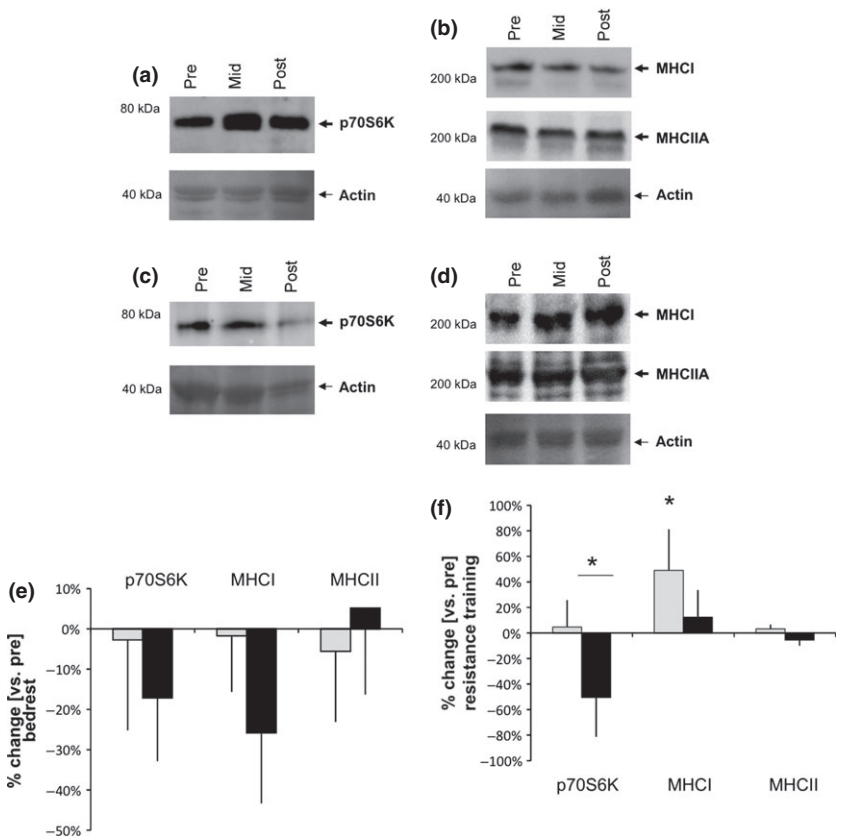

Fig. 1 Level alteration of factors related to muscle size with modified muscle loading. (a–d) Representative detection of p70S6K (a, c), and MHCI and MHCII (b, d) in Western blots of 20 μg total protein in muscle homogenate during the course of unloading (a, b) or overload (c, d). (e, f) Median and SE of changes in p70S6K, MHCI and MHCII protein levels per total protein during the time course of bedrest (b; $n = 9$) and resistance training (d; $n = 6$). * and + denote $P < 0.05$ and $0.05 \leq P < 0.10$ for the indicated comparison (Wilcoxon test). MHC, myosin heavy chain.

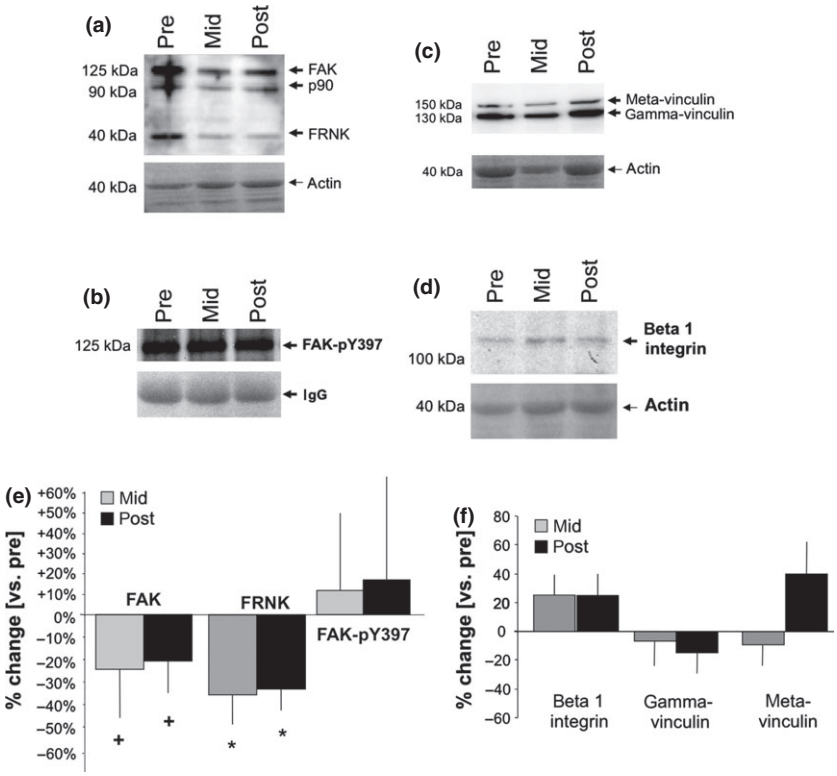

Fig. 2 Alterations in FAK in human vastus lateralis muscle with unloading. (a, d) Representative detection of FAK and FRNK (a), vinculin isoforms (c) and beta 1 integrin (d) in Western blots of 20 μg total protein, and pY397 phosphorylated FAK in immunoprecipitates from 1 mg soluble protein (b) of vastus lateralis muscle prior, mid- and post-bedrest. At the bottom of each panel, the respective loading control (i.e. sarcomeric actin or IgG on the Ponceau S stained membrane) is shown. (e, f) Median and SE of percentage changes in the content of FAK-related regulatory (e) and structural costamere proteins (f) per muscle protein during the time course of bedrest ($n = 9$). + and * denote $0.05 \leq P < 0.10$ and $P < 0.05$ vs. pre-intervention levels (Wilcoxon test). FAK, focal adhesion kinase.

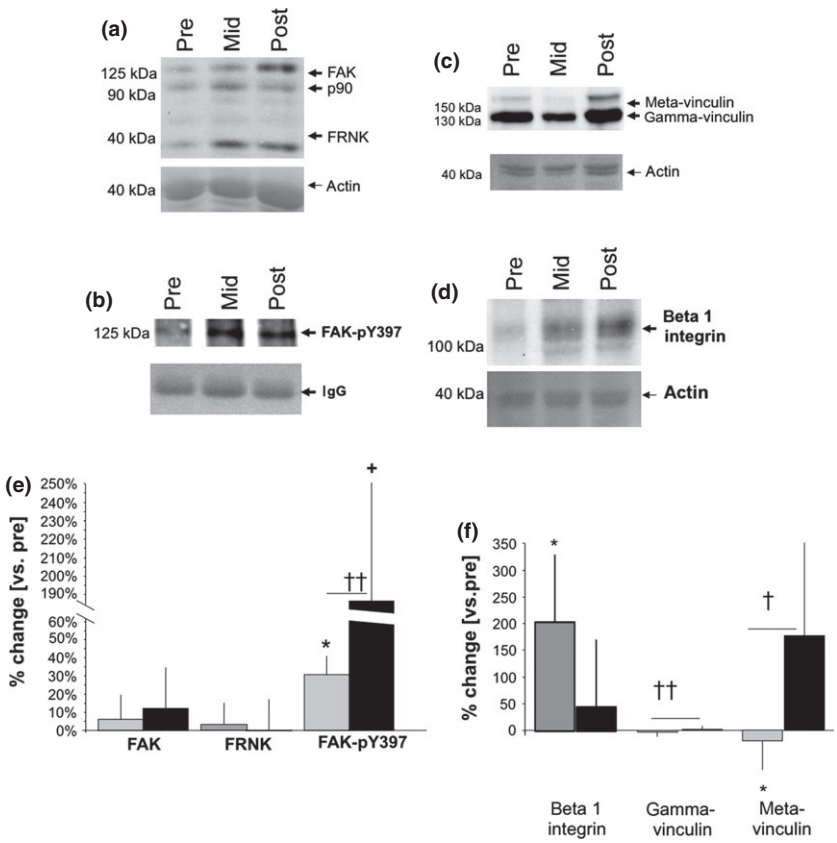

Fig. 3 Alterations in FAK in vastus lateralis muscle with increased loading. (a–d) Representative detection of FAK and FRNK (a), vinculin isoforms (c) and beta 1 integrin (d), and FAK-pY397 (b) in vastus lateralis muscle prior, mid- and post-resistance training. Respective loading controls are shown at the bottom of each panel. (e, f) Median and SE of changes in the content of FAK-related regulatory (e) and structural costamere proteins (f) per muscle protein during the time course of resistance training ($n = 6$). + and * denote $0.05 \leq P < 0.10$ and $P < 0.05$ vs. pre-intervention levels (Wilcoxon test). † and †† denote $0.05 \leq P < 0.10$ and $P < 0.05$, respectively, between fold changes mid- vs. post-intervention (Wilcoxon test). FAK, focal adhesion kinase.

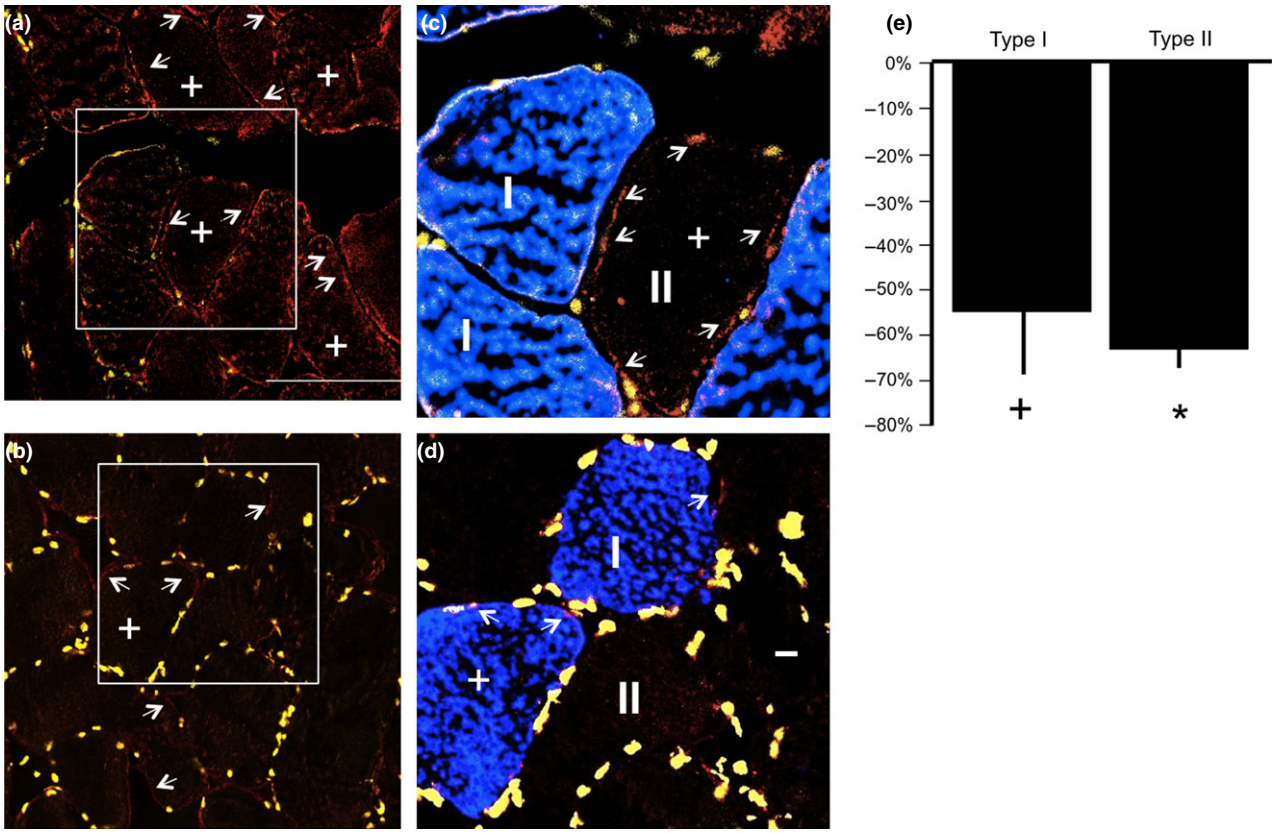

Fig. 4 Localization of FAK to the sarcolemma. (a, b) Example of a FAK-stained (red) section of vastus lateralis muscle in a subject before (a) and after (b) bedrest. Scale bar: 100 μm. (c, d) Enlarged images visualizing FAK signal (red) and type I MHC-stained muscle fibers (blue) pre- (c) and post-unloading in vastus lateralis muscle of the same subject (d). The frames in (a) and (b) indicate the region enlarged in (c) and (d), respectively. I, and II indicate examples of type I and type II muscle fibers. + and − denote examples of FAK-positive and negative muscle fibers. Examples of FAK-positive staining are indicated with white arrows. Nuclei appear in yellow. (e) Bar graph showing median + SE of unloading-induced changes in the percentage of type I, and type II, muscle fibers showing FAK-immunoreactivity. One-thousand and thirty fibers from four subjects were counted. * denotes $P < 0.05$ post- vs. pre-bedrest levels (paired *t*-test).

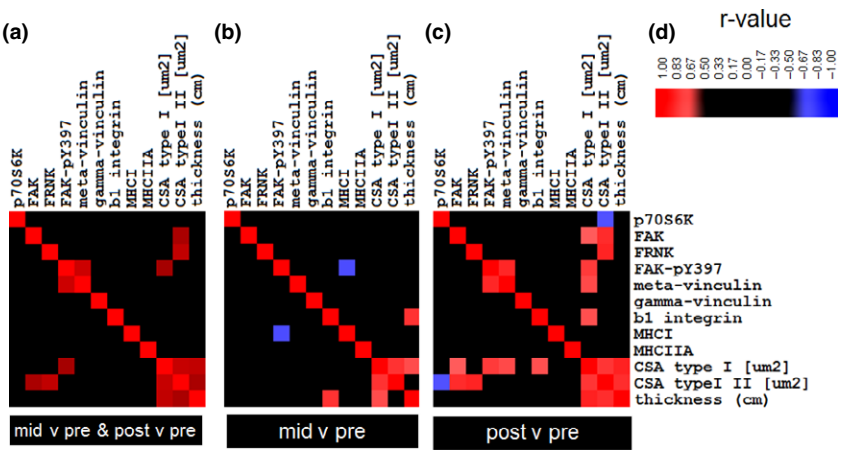

Fig. 5 Inter-relationship between muscular changes with altered loading. (a–c) Correlation matrix of changes in assessed parameters in a given phase, i.e. 'mid- vs. pre-' (a), 'post- vs. pre-' and 'mid- vs. post-' combined (b) and 'post- vs. pre-' (c) for the combined interventions. (d) *r*-values are given by color-coding, with those below 0.65 being blinded.

FOXO, NF-KB, are implicated in the control of muscle anabolism and catabolism (Wilkinson et al. 2008; McCarthy & Esser, 2010; Schiaffino & Mammucari, 2011), whereby p70S6K is a robust predictor for skeletal muscle growth (Baar & Esser, 1999; Terzis et al. 2008). The anatomical basis for load-dependent signal regulation of muscle hypertrophy and atrophy is currently poorly understood despite the widespread view that this issue is considered of great

importance (Adams et al. 2003; Chopard et al. 2009). Costameres, which reinforce the muscle fiber–extracellular matrix composite (Gullberg et al. 1998; Huijing, 1999), are prime candidates for the organization of muscle signaling as they represent sites where the mechanical stress of force transmission is integrated into signaling (Ervasti, 2003; Grounds et al. 2005). In order to address the role of costameres in the regulation of muscle size by mechanical loading, we assessed the association of level changes in the costamere's components and its upstream regulator, FAK, with changes in muscle thickness and CSA following bedrest and resistance training.

The present findings imply that the response to our resistance training paradigm involves an early and preferential hypertrophy of type I fibers. This is indicated by the tendency for an increase in the CSA of type I muscle fibers after just 10 training sessions (3 weeks) concomitantly with the selective increase in content of MHCI at this mid-point of resistance training (Fig. 1f; Table 2b). With completion of the second phase of training, when training-induced increases in CSA of type II fibers were larger than in the first phase of training, changes in MHCI protein content fell to pre-training levels (compare Fig. 1f with Table 2b). Myofibrils form the larger part of muscle material (Luthi et al. 1986). It follows that the absence of significant changes in MHCI content per total protein in the second phase of hypertrophy with resistance-type training reflects an increased incorporation of slow-type myofibrils being offset by elevations in CSA of type II myofibrils. This view is in line with slightly higher values of correlation between changes in muscle thickness and CSA of type I fibers compared with those of type II fibers. It remains to be assessed to what extent this difference in type I and type II fiber hypertrophy reflects the earlier time point of our study compared with other investigations (Williamson et al. 2001; West et al. 2010) and/or the contribution of slow concentric- and eccentric-type contractions in the employed training paradigm (Seynnes et al. 2007).

In line with our expectations, changes in the costamere regulator, FAK (Quach & Rando, 2006), were found to differ between bedrest and resistance-type training (Figs 2e and 3e). The downregulation of FAK protein levels with unloading, which was reflected by reduced FAK C-terminal immunoreactivity at the sarcolemma (Fig. 4D), reproduces our previous observations of load-regulated FAK expression and recruitment-related FAK staining at the sarcolemma (Gordon et al. 2001; Fluck et al. 2002; de Boer et al. 2007b; Evans et al. 2008). It is in line with previous findings regarding the non-significant reduction in total FAK content with 10 days of unilateral limb suspension (de Boer et al. 2007b). Interestingly, changes in the proxy of focal adhesion turnover in culture, i.e. FAK-pY397 content (Dumbauld et al. 2010), over all data points and at the end of resistance training correlated with changes in meta-vinculin ($r = 0.80$; 0.85), and both parameters demonstrated regulation with resistance training (Fig. 3e,f). These findings highlight the implication of modified fiber adhesion in muscle remodeling with altered loading in men, and point to a previously unrecognized implication of reduced content of the FAK inhibitor, FRNK, to this process (Fig. 2e). Specifically, the observations suggest a reduced capacity for FAK-mediated costamere assembly during bedrest.

Because myofibrillar mass largely contributes to total protein that serves as a reference for the estimation of total protein content, the increase in myofibrils with hypertrophy may camouflage (or dilute) any effect of resistance training on the amount of FAK. In this regard it is helpful to consider the geometric effects of atrophy and hypertrophy on the concentration of costameric proteins. As costameres reside at the fiber surface they scale to the fiber radius (i.e. $2\pi r$). By contrast, fiber volume that is the reference for total protein content is related to the square of the radius (i.e. πr^2). As a consequence, changes in fiber radius are expected to result in concentration changes of costameric protein per total protein that are inversely related to the change in fiber radius. Assuming that the expression of costamere components is not affected, the latter relationship is expected to result in negative correlations between individual changes in costamere proteins and in muscle (fiber) volume. This is in agreement with our finding of a close correlation ($r = -0.86$) between the changes in vastus lateralis thickness and meta-vinculin content mid-way into bedrest. As a consequence, the absence of a significant increase in the concentration of structural components of costameres with bedrest may indicate that their expression is affected, yet this does not manifest at the level of content vs. total protein. Conversely, hypertrophy induced by resistance training could have been expected to result in a reduction of the concentration of costameric proteins. However, we detected an increase, which further emphasizes that expressional adjustments of costameres are load-dependent.

The observed regulation of costamere components is of interest when put in the context of time course of changes in muscle (fiber) CSA with reduced and increased muscle loading. This comparison demonstrates that adjustments in the level of costamere components differ between the mid- and end-point of resistance training, and are matched to changes in muscle anatomy. These bi-phasic alterations in the costameric proteins, beta 1 integrin, gamma-vinculin and meta-vinculin, and the myofibrillar protein MHCI (Figs 1f 3f), are of significance given the concomitant (and larger) increase in muscle thickness and CSA of type I muscle fibers in the second phase of resistance training (Table 2). Thus, the observed changes in costamere component content and micro- and macroscopic estimates of hypertrophy were specifically inter-related during the first phase of resistance training. For instance, the changes in meta-vinculin, b1 integrin and FAK-pY397 content were correlated with changes in mean CSA of type I fibers with resistance

training ($r > 0.65$). We also noticed correlations between the changes in the costamere component meta-vinculin and changes in muscle thickness mid-way into resistance training ($r = 0.79$) and FAK with CSA post-resistance training (data not shown). In addition, beta 1 integrin and muscle thickness were correlated up to the mid-phase, but not the end, of the combined response to overload and unloading (*r*-value of 0.76 vs. 0.09). These differences emphasize that the upregulation of vinculin isoforms and of beta 1 integrin is associated with a marked hypertrophic response after the 10th vs. 27th session of resistance exercise. The latter finding relates to the reported dependence of fiber growth in culture on costamere-mediated attachment of nascent myofibrils (Quach & Rando, 2006; Konieczny et al. 2008). Taken together, these observations indicate that a possible structural reinforcement of costameres in the late phase of muscle hypertrophy contributes to the attachment of newly synthesized myofibrils after the integrin anchor is laid down.

The observation of an altered modality of muscle size regulation at the two time points of resistance training is supported by the reduced level of the serine/threonine kinase p70S6K in the second phase of training (Fig. 1c,d). It has been shown that post-translational activation of p70S6K is enhanced after different types of training in both the trained and untrained state (Wilkinson et al. 2008), and is positively correlated with the degree of hypertrophy with resistance training (Terzis et al. 2008). Interestingly, changes in p70S6K content correlated negatively with changes in CSA of type II fibers after altered loading ($r = -0.68$; Fig. 5), and changes in muscle thickness after resistance training ($r = -0.78$) and after bedrest ($r = -0.68$). To our understanding it is currently unknown whether signaling is maintained through the prolonged response to altered muscle loading. Our data now suggest that expressional regulation of p70S6K and costameres may contribute to muscle protein accretion during resistance training in men (Coffey et al. 2006).

It has been previously suggested that maintenance of nitric oxide signaling mechanisms and changes in protein turnover can be regarded as biomarkers for human skeletal muscle atrophy with long-term bedrest (Salanova et al. 2008). We show here that changes in costamere-associated muscle parameters, meta-vinculin, FAK-pY397 and MHCI, serve as indicators of muscle remodeling with altered loading in vastus lateralis muscle. This finding relates to the fiber type-specific localization of FAK and meta-vinculin to the sarcolemma (Fluck et al. 2002; Thoss et al. 2013). The observation is of interest, as one isoform of NO synthase as well is associated with costameres (Baum et al. 2000). Thus, our findings reinforce the notion that costamere components can serve as markers of load-dependent muscle remodeling.

A limitation of our study was that we did not quantify the localization of FAK at the sarcolemma as a function of muscle overload. This was due to an insufficient amount of high-quality bioptic sample to perform this characterization *post-hoc*. Also, we acknowledge that we did not assess an extensive list of costamere proteins for level alterations with increased and reduced muscle loading. Despite these constraints, we believe our findings provide novel information on the load-regulated content of structural (i.e. beta 1 integrin, vinculin and meta-vinculin) and regulatory factors of costameres in human subjects in relation to changes of muscle micro- and macro-structure.

Conclusion

Our observations point to a novel role for the organization of fiber adhesion in the mechano-regulation of muscle size in men. Notably, two phases of costamere remodeling with resistance training can be distinguished by the expression of the integrin anchor and vinculin isoforms, and CSA of muscle fibers, but not at the macroscopic level by muscle thickness.

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Author's contributions

Concept/design: MVN, MF; data acquisition: RL, RME, ORS, MVN, MF; data analysis/interpretation: ORS, MVN, MF; prepared figures: MF; drafting of manuscript: MF; editing and revision of manuscript: MVN, ORS, RME, MF; approved final version of manuscript: RL, RME, ORS, MVN, MF.

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