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Human skeletal muscle fibre contractile properties and proteomic profile: adaptations to 3-week unilateral lower limb suspension and active recovery

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Abbreviations ULLS, unilateral lower limb suspension; CSA, cross-sectional area; Po/CSA, specific force; Vo, shortening velocity; MHC, myosin heavy chain; MLC, myosin light chain; TnIf, troponin-I Fast; TnTf, troponin-T fast; Tpm1, Tropomyosin alpha chain; Act, actin; Myoz-1, Myozenin-1; CK, creatin kinase; ADK, adenylate kinase; TPI, triosephosphate isomerase; ENO, β-enolase; LDHA, lactate dehydrogenase A; NDUFS3, NADH dehydrogenase Fe-S protein 3; GSTP1, gluthatione S-transferase P; GSTM, gluthatione S-transferase M; CAH III, carbonic anhydrase-3; SOD1, Cu/Zn Superoxide dismutase; PRDX3, Peroxiredoxin-3; αβ-cry, αβ-crystallin; MuRF-1, muscle-specific ring finger protein-1; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; S6Rp, S6 Ribosomal protein; PGC-1α, peroxisome proliferative activated receptor-γ coactivator 1α; SREBP-1, Sterol regulatory element binding proteins-1; AMPK, AMP-activated protein kinase; NRF2, Nuclear factor (erythroid-derived 2)-like 2; FoxO, Forkhead box; ROS, reactive oxygen species.

Abstract

Following disuse muscle fibres function goes through adaptations such as loss of specific force (Po/CSA) and increase in unloaded shortening velocity (Vo), which could be due to both quantitative changes, i.e. atrophy, and qualitative changes in protein pattern. The underlying mechanisms have not been settled yet. In addition, little is known about the recovery of muscle mass and strength following disuse. Here we report an extensive data set describing in detail functional and protein content adaptations of skeletal muscle in response to both disuse and retraining. Eight young healthy subjects were subjected to 3 weeks unilateral lower limb suspension (ULLS), a widely used human model of disuse skeletal muscle atrophy. Needle biopsies samples were taken from the vastus lateralis muscle pre-ULLS, post-ULLS and after 3 weeks recovery during which heavy resistance training was performed (post-RT). After disuse, cross sectional area (CSA), specific force (Po/CSA) and myosin concentration (MC) decreased both in type 1 and 2A skinned muscle fibres. After recovery, CSA and MC returned to levels comparable to those observed before disuse, whereas Po/CSA and unloading shortening velocity reached a higher level. Myosin heavy chain (MHC) isoform composition of muscle samples did not differ among experimental groups. To study the mechanisms underlying such adaptations, a 2D proteomic analysis was performed. ULLS induced reduction of myofibrillar, metabolic (glycolytic and oxidative) and antioxidant defense system proteins content. Resistance training was very effective in counteracting ULLS induced alterations indicating that long term ULLS did not prevent the positive effect of exercise on human muscle.

Key points

- It is generally believed that muscle fibres go through atrophy following disuse with loss of specific force (Po/CSA) and increase in unloaded shortening velocity (Vo). However, the underlying mechanisms have not been settled yet.
- Most studies have focused on events during the developing of disuse, whereas the subsequent recovery phase, which is equally important, has been little studied
- Our findings support the hypotheses that specific force of muscle fibres decreased following ULLS and went back to normal following three weeks active recovery due to a loss and recovery of myosin and actin content
- Furthermore, muscle fibres went through extensive qualitative changes in muscle protein patter following ULLS, which were reversed by active recovery
- Resistance training was very effective in restoring both muscle mass and qualitative muscle changes indicating that long term ULLS did not prevent the positive effect of exercise on human muscle

Introduction

In disuse, muscle weakness and loss of power occur and have dramatic consequences on health and quality of life especially in the elderly population (Adams *et al.*, 2003; D'Antona *et al.*, 2003). Although the loss in muscle mass is well known to be the major cause of loss of force and power, it has become increasingly clearer that changes in muscle quality also play a very relevant role. In fact, loss of strength and power mostly exceeds the loss in muscle mass (Berg *et al.*, 1997; Fitts *et al.*, 2001; di Prampero & Narici, 2003).

Disuse can affect the whole body as in spaceflight or bed rest or be limited to one limb as following immobilization or unilateral lower limb suspension (ULLS). We have recently studied the adaptations of muscle proteome and of the underlying intracellular signalling pathways to bed rest as a model of whole body deconditioning (Brocca *et al.*, 2012). The adaptations of skeletal muscle to disuse and the underlying mechanisms widely vary through disuse models (Pellegrino *et al.*, 2011a). Here we study the changes in muscle quality following ULLS, which has been successfully used to induce disuse atrophy in one limb for decades.

Muscle fibres themselves have been found to go through not only atrophy, but also qualitative changes following disuse. A disproportionate loss of force compared to CSA, i.e. a loss of specific force (Po/CSA) (Larsson *et al.*, 1996; Widrick *et al.*, 1999b; Widrick *et al.*, 2002b), and an increase in unloaded shortening velocity (Vo)(Widrick *et al.*, 1997; Widrick *et al.*, 1999a; Widrick *et al.*, 2001) have been observed. However, contradictory findings have been reported (Widrick *et al.*, 1997; Widrick *et al.*, 2002b; Trappe *et al.*, 2004; Canepari *et al.*, 2010; Fitts *et al.*, 2010) and there is no general consensus on the consequences of disuse on muscle fibres structure and function. Moreover, in most works so far, in vivo and ex vivo analysis have been performed on different subjects groups hindering the possibility to relate adaptations at individual muscle fibre level studied ex vivo with those occurring in whole muscles in vivo.

Importantly, the mechanisms underlying the potential loss of Po/CSA and increase in Vo at muscle fibre level have not been settled either. A decrease in myosin concentration within individual muscle fibres and therefore a decrease in the number of force developing cross-bridges has been suggested as a potential cause of Po/CSA loss (D'Antona *et al.*, 2003; Borina *et al.*, 2010). However, no analysis of Po/CSA and myosin concentration of muscle fibres pre- and post-disuse in young healthy subjects has been performed yet. Moreover, recently, it has been suggested that impairment of muscle fibres specific force and power due to disuse in elderly subjects could depend on altered acto-myosin kinetics in turn caused by post-translational modifications of myosin (Callahan *et al.*, 2014).

The observations of a decrease in myosin concentration and of myosin post-translational modifications highlight the potential occurrence of qualitative adaptations of the whole muscle protein pattern and their potential functional relevance in disuse. In bulk vastus lateralis muscle samples, protein fractions of myosin and actin were found to be unchanged following 35 days of ULLS notwithstanding significant atrophy (9%)(Carrithers *et al.*, 2002; Haus *et al.*, 2007). On the contrary, several evidences indicated a loss of myofibrillar proteins and a profound alteration in their relative content in muscle fibres (Riley *et al.*, 2002; Haddad *et al.*, 2005; Borina *et al.*, 2010). Importantly, recent time course analysis of muscle proteome of vastus lateralis following bed rest has confirmed an early and persistent down-regulation not only of many myofibrillar proteins (Brocca *et al.*, 2012), but also of several other functional groups of proteins, e.g. metabolic enzymes and antioxidant defence systems, underlying the complexity of the qualitative adaptations in muscle protein pattern to disuse and the relevance of a global proteome analysis.

Most studies have focused on events during the developing of disuse, whereas the subsequent recovery phase, which is equally important, has been little studied (Berg *et al.*, 1991; Berg & Tesch, 1996; Suetta *et al.*, 2009). Hvid and collaborators have shown that 4 days of lower limb disuse led to marked decrements in knee extensor mechanical muscle function and 7 days of recovery completely restore mechanical function in young individuals (Hvid *et al.*, 2014).

We recently showed that, the loss in isometric torque (26%) and quadriceps volume (10%) and the decrease in fascicle length and pennation angle which occurred following 3 weeks ULLS were fully restored through active recovery (Campbell *et al.*, 2013). However, it is still unclear whether and to which extent the recovery of in vivo muscle function and muscle architecture was paralleled by the recovery of single muscle fibres structure and function, and of muscle protein pattern as such adaptations have not been studied following recovery.

The general goal of the present study was to clarify the nature of the qualitative changes of skeletal muscle following disuse and rehabilitation and the underlying mechanisms. The study had two specific and strictly interrelated aims: - to define the adaptations in CSA, specific force and unloaded shortening velocity of identified types of single muscle fibres to disuse and retraining and to clarify the underlying mechanisms; -to define the adaptations of the global muscle protein pattern to disuse and recovery. The study could take advantage of the availability of muscle samples from the same population of young healthy subjects, which we studied in vivo as regards muscle function, size and architecture. The adaptations at cellular and molecular level could, therefore, be related to in vivo ones.

Methods

Subjects

Eight healthy young men, aged 18-25 years, with no previous history of traumatic lesions or muscular diseases were enrolled in the study and subjected to a three-week ULLS program followed by a three-week resistance training program. All participants signed a written informed consent and the study was approved by the Manchester Metropolitan Faculty of Science and Engineering Ethics Committee and conformed to the principles of the Declaration of Helsinki on human experimentation.

ULLS

In ULLS, the treated limb is flexed and suspended above the ground by the use of a shoulder harness. The procedure reduces many of the expenses and logistical problems associated with other unloading conditions (bed rest or space flight) (Hackney & Ploutz-Snyder, 2012). Moreover, confining disuse muscle atrophy to the unloaded limb, it closely resembles joint unloading following muscle and skeletal injuries, a very frequent and relevant condition in clinical practice.

During the study, participants were monitored every two days by measuring plasma D-dimer concentration and performing colour coded Doppler sonography in order to detect deep venous thrombosis (DVT) development in the early sub clinical phase.

This study was conducted using the same precautions followed with success by our group in a previous ULLS study (De Boer *et al*. 2007).

ULLS causes a loss of muscle mass, similar to other disuse models of approximately 0.44% per day in the vastus lateralis, which is among the most affected muscles (Hackney & Ploutz-Snyder, 2012)

RESISTENCE TRAINING PROTOCOL

Following the ULLS, participants attended the laboratory 3 days per week for a period of 3 weeks to undergo a resistance training (RT) program. Quadriceps extensions were performed at 80 % of participants' one repetition maximum (1RM). The 1RM was assessed weekly and the resistance level was adjusted accordingly. Participants first warmed up with a series of sub maximal contractions, after which the resistance was increased to find the maximum. The training load was set at 80 % of 1RM, and three sets of ten contractions were performed during each training session using a Technogym® knee extension machine.

Muscle biopsies

Using a procedure previously described (Bergstrom, 1979; Bottinelli *et al.*, 1996), three needle biopsies were taken from vastus lateralis muscle of all subjects: one pre suspension (Pre ULLS), one post suspension (Post-ULLS), one post exercise recovery (Post-RT). Muscle biopsies were divided in several portions, and a tissue sharing approach among several laboratories collaborating in the MYOAGE consortium, supported by European Community, was used to optimize, for ethical and economic reasons, the burden on the volunteers. Two, 20 mg, portions were used in the present work. One was stored at -20°C in a skinning solution plus glycerol (50% v/v) and used for functional analysis of individual muscle fibres. One was frozen in liquid nitrogen and stored at -80°C for proteomic, gene expression and MHC isoform distribution analysis. For technical reasons, two glycerinated Post-ULLS biopsies deteriorated during shipping at -20°C from Manchester, where they were taken, to Pavia, where analysis was performed, and could not be used for functional analysis of individual muscle fibres. Therefore, only fibres from 6 subjects were included in the analysis of force and unloaded shortening velocity. Proteomic analysis was performed on muscle samples stored and shipped at -80°C from all 8 subjects.

Single fibres analysis

Cross-sectional area (CSA), force and maximum shortening velocity of single muscle fibers were analyzed as previously described in detail (Bottinelli *et al.*, 1996). Briefly, segments of single fibers were manually isolated from muscle bundles with the help of a stereomicroscope at 20-40X magnifications in a muscle chamber containing skinning solution (K-p 150 mM, KH2PO4 5mM, MgAc 5 mM, DTT 1 mM, EGTA 5 mM, Na2-ATP 3 mM, pH 7, Leupeptine Hydrochloride 20µg/ml, E64 10 µM). The fibers were immersed for 1 h in skinning solution containing 0.1% Triton X-100 and afterward returned to the previous skinning solution. Each fiber was then mounted between two hooks on a stage of an inverted microscope. Isometric force (P_0) and unloaded shortening velocity (V_0) were measured by the slack test technique placing each fiber in activating solution: 100 mm KCl, 20 mm imidazole, 5 mm MgCl2, 5 mm Na2ATP, 5 mm EGTA, 25 mm creatine phosphate, 300 U ml−1 creatine kinase, Leupeptine Hydrochloride 20µg/ml, E64 10 µM. Experiments were performed at 12°C, in conditions of maximal activation (pCa 4.5) and at optimal sarcomere length for force developing (Bottinelli & Reggiani, 2000; D'Antona *et al.*, 2003).

For each fibers, length and diameters were measured in the same experimental apparatus; the width and the depth of every fiber was determined at 320x magnification on 10 different positions on the fiber, rotating the hooks. Assuming fiber section as an ellipse, the CSA was calculated and then the

volume was estimated by multiplying CSA and length. Fibers were finally characterized on the basis of MHC isoform.

Myosin concentration analysis

Myosin content analysis was performed using an approach previously described in detail (D'Antona *et al.*, 2003; Borina *et al.*, 2010) with some modifications. After mechanical experiment, the fibers were placed in 30 µl of lysis buffer (Laemmli, 1970) at 4 C for 18 h to complete myosin extraction. Subsequently, for each fiber 10 and 20 µl of lysis buffer in which the fiber segment was dissolved, were loaded on 12% linear polyacrylamide gel, run at 16mA for 4h at 4°C and stained with Colloidal Coomassie (Gelcode Blue Stain Reagent – Pierce). In the same gel, a known amount of myosin standard were loaded in order to determine a standard curve.

Gels were then imaged using a high-resolution scanner (Epson Expression 1680 Pro). The brightness–area product (BAP) of the myosin bands was determined using the software Adobe Photoshop CS5 (Adobe). BAP correspond to product of the number of pixels of the whole MHC band for the mean intensity level of the pixels. The myosin concentration standard curve was built plotting together BAP values from myosin standard and known amount of myosin loaded. Finally BAP values evaluated for single fiber were inserted in the standard curve obtaining their myosin concentration.

Analysis of myosin heavy chain isoform content

Separation and identification of myosin heavy chain (MHC) isoforms in single fibers and in the whole biopsy was performed as previously described (Bottinelli *et al.*, 1996; D'Antona *et al.*, 2003). Single muscle fiber segments used for mechanical experiments and a part of the biopsy were dissolved in Laemmli solution (Laemmli, 1970), and loaded on 6% SDS-PAGE polyacrylamide gels. Electrophoresis was run overnight at 100 V; following silver stain, three bands were separated in the region of MHC isoforms. In relation to the presence of one or two bands in the MHC region, single fibers can be classified in one of the following types: 1, 2A, 2X (pure fibers) and 1–2A, 2A–2X (mixed fibers). Densitometric analysis of MHC bands was performed to assess the relative proportion of MHC isoforms in the muscle biopsies (Pellegrino *et al.*, 2003).

Proteome analysis (2-DE)

Sample preparation

Muscle samples, were prepared with the same procedures used previously (Brocca *et al.*, 2010). Frozen portion of biopsy was pulverized in a steel mortar with liquid nitrogen to obtain a powder that was immediately resuspended in a lysis buffer (8 M urea, 2 M thiourea, 4% Chaps, 65mM DTT

and 40 mM Tris base). The samples were vortexed, frozen with liquid nitrogen, thawed at room temperature four times, incubated with DNase and RNase for 45min at 4◦C to separate proteins from nucleic acids and finally spun at 18000 g for 30 min. Protein concentration in the dissolved samples was determined with a protein assay kit (2D quant Kit, GE Healthcare). A sample mix was obtained for each experimental group (Pre-ULLS, Post-ULLS and Post-RT). Sample mix contained an equal protein quantity taken from each muscle sample of Pre-ULLS, Post-ULLS and Post-RT.

Two-dimensional electrophoresis.

First dimension, isoelectrofocusing, was carried out using IPGphor system (Ettan IPGphor isoelectric Focusing Sistem - GE Healthcare). 150 µg protein were loaded on IPG gel strips, pH 3-11 NL (non linear) 13 cm, that were rehydrated for 14h, at 30 Volt and at 20°C, in 250 µl of reswelling buffer (8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.1% (v/v) tergitol NP7 (Sigma), 65 mM DTT, 0.5% (v/v) pharmalyte 3-11NL (GE Healthcare)). Strips were focused at 20000 Vhr, at constant temperature of 20°C and limiting the current to 50 µA per IPG gel strip. After isoelectrofocusing the strips were stored at -80°C until use or equilibrated immediately for 10-12 min in 5 ml of equilibration buffer (50 mM Tris pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 3% (w/v) iodoacetamide). Then, the immobiline IPG gel strips were applied to 15% T, 2.5% C polyacrylamide gels without a stacking gel. The separation was performed at 80V for 17h at room temperature. 2D gels were fixed for 2h in fixing solution (ethanol 40% (v/v) acetic acid 10% (v/v)), stained with fluorescent staining (FlamingoTM Fluorescent Gel Stain by BIO-RAD) for 3h and destained with 0.1% (w/v) Tween 20 solution for 10 minutes.

Triplicate gels of each sample group were obtained, visualised using a Typhoon laser scanner (GE Healthcare) and analysed with Platinum Software (GE Healthcare). For the analysis, we have considered only spots present in all gels. The software provided normalised volume for each spot (representing protein amount). The volumes of each spots in the triplicate gels were averaged and spots statistically changed were obtained ($P < 0.05$).

Protein Identification by Mass Spectrometry (MS) and Database Searching.

Mass spectrometry was performed as previously described (Brocca *et al.*, 2010). 2D gels were loaded with 300 µg of proteins per strip and the electrophoretic run was carried out with the same conditions described above. After staining with Colloidal Coomassie, spots of interest were excised from the gel, destained (100 mM ammonium bicarbonate (ABC) and 50% (v/v) acetonitrile (ACN)) for 45 min at 22°C and dried (100% ACN for 15 min). Protein spots were then reduced with 25 mM ABC containing 10 mM DTT for 1 h at 60°C and then alkylated with 55 mM iodoacetamide in 25 mM ABC for 30 min in the dark at 22°C. Gels pieces were washed and dried using 100% ACN for

10 min. After the dehydratation for 1 h at 60°C, gel pieces were incubated with 13 µl of sequencing grade modified trypsin (Promega, USA; 12.5 µg/ml in 40 mM ABC with 10% ACN, pH 8.0) overnight at 40°C (Shevchenko *et al.*, 1996). After digestion, peptides were washed with 30 µl of 25 mM ABC, shrunk with 100% ACN and extracted twice with a mixture of 50% ACN–5% formic acid (FA). For MS and MS/MS MALDI analysis, peptides were redissolved in 4 µl of alpha-CHCA (2,5 mg/ml in 70% ACN-0.1% TFA). 1.5 µl of each sample was spotted directly onto a dry MALDI plate (ABSciex, Foster City, CA, USA). Peptides on MALDI plate were then desalted with a cold solution of 10mM ammonium phosphate and 0.1% TFA. The analysis of samples was performed using a MALDI-TOF-TOF 4800 mass spectrometer (ABSciex). Spectra acquisition and processing was performed using the 4000 series explorer software (ABSciex) version 3.5.28193 in positive reflectron mode at fixed laser fluency with low mass gate and delayed extraction. Peptide masses were acquired by steps of 50 spectra for the range of 900 to 4000Da. MS spectra were summed from 500 laser shots from an Nd-YAG laser operating at 355 nm and 200Hz. After filtering tryptic-, keratin- and matrix-contaminant peaks up to 15 parent ions were selected for subsequent MS/MS fragmentation according to mass range, signal intensity, signal to noise ratio, and absence of neighboring masses in the MS spectrum. MS/MS spectra were acquired in 1 kV positive mode and 1000 shots were summed by increment of 50. Database searching was carried out using Mascot 2.2 (MatrixScience, London, UK) combining MS and MS/MS interrogations on human from SwissProt databank (20 321 sequences, March 2012, www.expasy.org). Positive identification was based on a Mascot score above the significance level (i.e. <5%). MS and MS/MS ORBITRAP analyses were realized using an Ultimate 3000 Rapid Separation Liquid Chromatographic (RSLC) system (Thermo Fisher Scientific) online with a hybrid LTQ-Orbitrap-Velos mass spectrometer (Thermo Fisher Scientific). Briefly, peptides were loaded and washed on a C18 reverse phase precolumn (3 µm particle size, 100 Å pore size, 150 µm i.d., 1 cm length). The loading buffer contains 98% H2O, 2% ACN and 0.1% TFA. Peptides were then separated on a C18 reverse phase resin (2 µm particle size, 100 Å pore size, 75 µm i.d., 15 cm length) with a 4 min “effective gradient” from 100% A (0.1% FA and 100% H2O) to 50% B (80% ACN, 0.085% FA and 20% H2O). The Linear Trap Quadrupole Orbitrap mass spectrometer acquired data throughout the elution process and operated in a data dependent scheme with full MS scans acquired with the Orbitrap, followed by up to 20 LTQ MS/MS CID spectra on the most abundant ions detected in the MS scan. Mass spectrometer settings were: full MS (AGC: 1*106, resolution: 6*104, m/z range 400-2000, maximum ion injection time: 500 ms); MS/MS (AGC: 5*103, maximum injection time: 50 ms, minimum signal threshold: 500, isolation width: 2Da, dynamic exclusion time setting: 15 s). The fragmentation was permitted of precursor with a charge state of 2, 3, 4 and up. For database searching, all the search

parameters were the same as MALDI search except the precursor mass tolerance which was set to 5 ppm and the fragment mass tolerance to 0.45 Da.

Immunoblot analysis.

Some spot showing expression changes with proteomic analysis, and catalase, that do not appear in 2D maps, were subsequently tested by comparative immunoblotting analysis as previously described (Brocca *et al.*, 2012). About 20 µg of muscle samples, prepared and used for 2D electrophoresis, were loaded on Any kD precast polyacrylamide gel (Biorad product). The proteins were electrotransferred from gels to nitrocellulose membranes at 100V for 2h and the Western blot analysis was performed. Nitrocellulose membranes were blocked in 5% milk in TBST (Tris 0.02M, NaCl 0.05 M pH 7.4-7.6, 0.1% Tween20) for 1h and then incubated in primary antibody at 4°C overnight. The membranes were probed with antibody specific to Catalase (rabbit-anti Catalase, Abcam), Triosephosphate isomerase (mouse-anti Trioseph isom, Abcam), beta-enolase (mouse-anti β-enolase, Abnova), Lactate dehydrogenase (rabbit-anti LDH, Abcam), Superoxide Dismutase 1 (rabbit-anti SOD1, Abcam), Peroxiredoxin 3 (mouse-anti PRDX3, Abcam), Heat Shock Protein B1 (mouse-anti Hsp27, Abcam), α-β crystallin (rabbit-anti α-β crystallin, Abcam), Aldolase A (mouse-anti aldolase A, Abcam). After several rinses in TTBS (0.1% Tween-20 in TBS), the membranes were incubated in HRP-conjugated secondary antibody, rabbit-anti-mouse (Dako) or goat-anti-rabbit (Cell Signalling) for 1h at room temperature. The protein bands were visualized by an enhanced chemiluminescence method (ECL Advance, GE Healthcare product). The content of single protein investigated was assessed by determining the brightness-area product (BAP) of the protein bands.

Some proteins involved in muscle protein synthesis signaling pathway were investigated by western blotting analysis and the muscular samples from each sample group were probed with specific antibodies. For this purpose muscle samples (stored at -80°C), were pulverized with liquid nitrogen and immediately resuspended in a lysis buffer [20mM TRIS-HCl, 1% triton x100, 10%Glycerol, 150mM NaCl, 5 mM EDTA, 100mM NaF and 2mM NaPPi supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis MO) and 1mM PMSF]; lysis tissue was performed on ice for 20 minutes and the homogenate obtained was centrifuged at 18000 g for 20 min at 4°C and the supernatant was transferred to a clean eppendorf tube and stored at -80°C until ready to use. Protein concentration was determined using the RC DC^{TM} protein assay kit (Biorad product). 40 µg of muscle samples were added at the loading buffer (8% SDS, 20% 2-mercaptoehtanol, 40% glycerol, bromophenol blue traces, 0.25 M Tris HCl, pH 6.8) and finally loaded on Any kD precast polyacrylamide gel (Biorad product). The proteins were

electrotransferred to nitrocellulose membranes at 35mA overnight. After block in 5% milk in TBST for 1h, the membranes were incubated overnight in primary antiboby diluted with 5% milk or 5% BSA (only for the phosphor-specific antibodies) in TBST. The membranes were probed with antibody specific to Akt (rabbit-anti Akt, Millipore), p-Akt (ser 473)(rabbit-anti p-Akt, Millipore), 4EBP1 (rabbit-anti 4EBP1, Cell Signalling), p4EBP1 (rabbit-anti p4EBP1, Cell Signalling), S6Rp (rabbit-anti S6Rp, Cell Signalling), p-S6Rp(ser235/236) (rabbit-anti p-S6Rp, Cell Signalling), AMPK (rabbit-anti AMPK, Cell Signalling), p- AMPK(thr 172) (rabbit-anti p- AMPK, Cell Signalling). Finally the membranes were incubated in HRP-conjugated secondary antibody, goat-anti-rabbit (Millipore), diluted in 5% milk, for 1 h at room temperature; the protein bands were visualized by an enhanced chemiluminescence (ECL Advance, GE Healthcare product) and the protein content were determined by the brightness-area product (BAP) of the protein band normalized to the tubulin.

RT-PCR analysis.

RT-PCR analysis has been carried out as previously described (Cannavino *et al.*, 2014). Total RNA, from skeletal samples, were extracted using the Promega SV Total RNA isolation kit; the concentration of RNA were evaluated by using NanoDrop instrument (Thermo scientific). 300 nr of RNA were reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen) to obtain cDNA. The cDNA was analyzed by RT-PCR with the SYBR Green PCR kit (Applied Byosistem) and the data were normalized to β2-microglobulin expression. Forward (FP) and reverse (RP) primers used for RT-PCR are listed below:

MuRF-1 FP: 5'–CCTGAGAGCCATTGACTTTGG–3', RP: 5'–CTTCCCTTCTGTGGACTCTTCCT–3'; *Atrogin-1* FP: 5'-GCAGCTGAACAACATTCAGATCAC–3', RP: 5'-CAGCCTCTGCATGATGTTCAGT-3'; *p62* FP: 5'–GCTTCCAGGCGCACTACC–3', RP: 5'–CATCCTCACGTAGGACATGG–3'; *Beclin-1* FP: 5'–TGGAAGGGTCTAAGACGT–3', RP: 5'–GGCTGTGGTAAGTAATGGA–3'; *PGC1alpha* FP: 5'-CAGGATTTCATCTGAGTGTGGA-3', RP: 5'-GCGAGAGAGAAAGGAAAAGAACAA-3'; *SREBP-1* FP: 5'–CGTCCCTCCTGTTGTGAAAT–3', RP: 5'–AGTGTTTTCACGGGACCAAG–3'; *NRF2* FP: 5'-CACAGAAGACCCCAACCAGT-3', RP: 5'-CTGTGCTTTCAGGGTGGTTT-3'; *β2-microglobulin* FP: 5'-GCTGTGCTCGCGCTACTCTCTCT-3', RP: 5'-TCTGCTGGATGACGTGAGTAAAC-3'.

Carbonylated proteins.

Carbonylated proteins were analysed based on the method previously reported (Brocca *et al.*, 2010). Frozen samples from each subject group were suspended in a lysis buffer (50 mM Tris-HCl pH7.6, 250 mM NaCl, 5 mM EDTA protease inhibitor cocktail and phosphatase inhibitor cocktail), left on ice for 20 minutes and finally centrifuged at 18000 g for 20 min at 4°C. Protein concentration was determined using the RC DC^{TM} protein assay kit (Biorad product).

The protein carbonylation level was detected using the OxyBlot™ Kit (Millipore) that provides reagents for sensitive immunodetection of carbonyl groups. Carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNP) by reaction with 2,4-dinitrophenylhydrazine (DNPH). In detail, 10 μg of proteins for each muscle sample were denatured with SDS solution at a final concentration of 6%. The DNPH solution was added to obtain the derivation; the reaction was stopped after 15 min of incubation at room temperature. The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis (Anykd Biorad gels) followed by Western blotting. Proteins were transferred to nitrocellulose membranes at 100V for 2h, stained with Ponceau Red (Sigma) and then scanned. The membranes were blocked by incubation with 3% bovine serum albumin (BSA) for 1 h; then the membranes were incubated with rabbit anti-DNP antibody for 1h at room temperature and subsequently with a horseradish peroxidase-antibody conjugate (goat anti-rabbit IgG). The membranes are treated with chemiluminescent reagents (ECL advance as describe previously). The positive bands emitting light were detected by short exposure to photographic films.

The oxidative status of Pre-ULLS, Post-ULLS and Post-RT group was analyzed quantitatively by comparison of the signal intensity of immune-positive proteins normalized on total proteins amount loaded on gels (ponceau staining signal)

MLC phosphorylation analysis.

The same samples used for proteomic analysis were utilized to assess myosin light chain (MLC) phosphorylation. In this regard 20 μg of proteins were delipidated and desalted adding methanol and chloroform following by centrifuge at 18000 g for 5 min, in order to allow an adequate separation by electrophoresis and subsequent staining by phosphoprotein gel stain (Pro-Q®Diamond, life technologies). The proteins were loaded on Any kD precast polyacrylamide gel and the electrophoresis was run at 100 V for 1.5 h. The gel was immersed in fix solution (50% methanol and 10% acetic acid) overnight and then washed three times for 10 minutes in ultrapure water to remove all of the methanol and acetic acid from the gel. The gel was incubated in Pro-Q®Diamond phosphoprotein gel stain in gentle agitation for 90 minutes, then destained in destain solution (20% acetonitrile, 50 mM sodium acetate, pH 4.0) for reducing gel background and washed twice with ultrapure water. Finally MLC bands were visualized using a Typhoon laser scanner (GE Healthcare) and analyzed with Platinum Software (GE Healthcare). The same gel was also stain with Coomassie blue in order to analyses the total MLC content. MLC phosphorylation was assessed normalizing MLC phosphorylation degree on total MLC content.

Statistical analysis.

Data were expressed as mean ± S.E.M. Statistical significance of the differences between means was assessed by one-way ANOVA followed by Student-Newman-Keuls test. A probability of less than 5% was considered significant ($P<0.05$).

Results

Muscle biopsy samples were divided in three parts: one was put in skinning solution and glycerol and used for single muscle fibre analysis; two were immediately frozen in liquid nitrogen and used for proteomic and intracellular signalling analyses.

Fibre size, force and unloaded shortening velocity of single muscle fibres

For each subject 25 single muscle fibres were analysed. Mean values of CSA of identified types of individual muscle fibres Pre-ULLS, Post-ULLS and Post-RT, determined in the population of fibres used for functional analysis, are reported in Fig 1A. The analysis focused on pure type 1 and 2A muscle fibres whose percentage number was the following: type 1, Pre-ULLS 36%, Post-ULLS 46%, Post-RT 29%, type 2A, Pre-ULLS 40%, Post-ULLS 40%, Post-RT 41%. Hybrid (type 1-2A Pre-ULLS n=6, Post-ULLS n=1, Post-RT n=4 and 2AX Pre-ULLS n=10, Post-ULLS n=4, Post-RT n=27) fibres were discarded as their force and velocity could be affected by the presence of more than one MHC isoform with different functional properties. Type 2X fibres (Pre-ULLS n=0, Post-ULLS n=0, Post-RT n=6) were too few to provide statistically significant comparisons. CSA of type 1 fibres was lower (-22%) Post-ULLS than Pre-ULLS and recovered (+17%) Post-RT compared to Post-ULLS. CSA of type 1 fibres post-RT was not significantly different from CSA of type 1 fibres pre-ULLS, i.e. recovery was complete. Type 2A fibres showed the same extent of atrophy as type 1 (-23%) after suspension and no complete recovery (+8%) after retraining.

Po/CSA was lower in both type 1 (-13%) and type 2A (-16%) fibres Post-ULLS (Fig. 1B) than Pre-ULLS. The recovery of Po/CSA following RT was very large in both type 1 (+50%) and type 2A (+37%) compared to Post-ULLS overcoming the Pre-ULLS values (Fig. 1B).

Vo was unchanged Post-ULLS in both fiber types, whereas it was higher in both type 1 (+32%) and in type 2A (+53%) Post-RT than Post-ULLS (Fig. 1C).

Adaptations in muscle proteins

<u>MHC, MLC isoform distribution, myosin/actin ratio and myosin concentration in individual muscle fibres</u>

MHC and MLC isoform composition were determined in biopsy samples from Vastus lateralis muscle of all subjects from each groups. No significant differences were found in any condition both in MHC (Fig. 2A) and MLC (Fig. 2B) isoform distribution. Only some non-significant trend was observed, among which a higher MHC-2A content Post-RT than Pre-ULLS.

Myosin concentration was evaluated in all individual muscle fibres whose CSA, Po/CSA and Vo are reported. Myosin concentration was significantly lower Post-ULLS than Pre-ULLS in both fibre types (-26% for type 1 fibres and -30% for type 2A fibres) and showed a complete recovery after retraining (Fig. 2C). Interestingly although Po/CSA was higher Post-RT than Pre-ULLS (Fig. 1B), myosin concentration was not. The ratio between myosin and actin content (M/A) was determined by densitometry of the two proteins bands. The M/A ratio was significantly decreased after disuse and returned to the Pre-ULLS level after exercise (Fig.2D).

Proteomic analysis

Proteomic maps of vastus lateralis muscle, for each experimental condition, were obtained using 2D gel electrophoresis. In each 2D-gel more than 800 protein spots were detected and analysed for differential expression. Differentially expressed protein spots were subsequently identified by MALDI-Tof (Table 1). Identified proteins were grouped based on their functional role in the following categories: myofibrillar proteins, metabolic enzymes (oxidative, glycolytic, creatine kinase, adenylate kinase), antioxidant defense systems and other proteins. Identified spots are indicated in the 2D map shown in Fig. 3.

A general trend in the adaptations of protein pattern was observed: a proteins down-regulation in Post-ULLS respect to Pre-ULLS and a proteins up-regulation in Post-RT respect to Post-ULLS.

Among myofibrillar proteins, 5 spots identified as troponin-I Fast (TnIf), one spot identified as troponin-T fast (TnTf), one spot as myosin light chain 2s (MLC-2s) and one as myozenin-1 were significantly down-regulated Post-ULLS vs Pre-ULLS, and a trend for a lower content of tropomyosin and actin was observed (Fig. 4). Post-RT, the content of the same proteins was up-regulated towards the Pre-ULLS levels. None of the proteins significantly down-regulated Post-ULLS vs Pre-ULLS was still significantly down-regulated Post-RT vs Pre-ULLS, but only 3 were significantly up-regulated Post-RT vs Post-ULLS (tropomyosin, MLC-2s and actin). One spot corresponding to MLC-2s was significantly up-regulated also Post-RT vs Pre-ULLS.

Several expression adaptations in metabolic enzymes were identified following ULLS and resistance training. After disuse, a general down regulation in glycolytic enzymes, in two oxidative enzymes and in two enzymes involved in the balance between ATP, ADP and AMP, creatin kinase (CK) and adenylate kinase (ADK), was observed (Fig. 5). Spots relative to triosephosphate isomerase (TPI), β-enolase (ENO), lactate dehydrogenase A (LDHA), CK and ADK were statistically down-regulated Post-ULLS vs Pre-ULLS. Post-RT, all such proteins, but two (CK and ADK), showed a trend for a recovery towards Pre-ULLS levels and most of them were, indeed,

significantly higher Post-RT vs Post-ULLS. Both spots of CK did not recover, but were even lower Post-RT vs Pre-ULLS and only one of the two ADK spots recovered Post-RT. Of the four LDHA spots three were down-regulated Post-ULLS vs Pre-ULLS. The one that was not down-regulated Post-ULLS, was three fold up-regulated Post-RT vs Pre-ULLS. Some key spots showing expression changes with the proteomic analysis were subsequently tested by comparative immunoblotting in order to confirm their variations. Among energy production systems the approach was applied to Triosephosphate isomerase, β-enolase, LDHA, and Aldolase A (Fig.7). The results confirmed the behaviour described by proteomic analysis.

Antioxidant defence systems were a major functional group differentially expressed following disuse and rehabilitation (Fig.6A). A significant down regulation in gluthatione S-transferase P (GSTP1), in carbonic anhydrase-3 (CAH III), in two spots of α-β-crystallin and in one spot of HSPB1 was detected post-ULLS vs pre-ULLS, whereas Cu/Zn Superoxide dismutase (SOD1) and Peroxiredoxin-3 (PRDX3) were up-regulated. In Post-RT a general up-regulation of antioxidant defence systems was observed with respect to Post-ULLS, with the exception of PRDX3 and gluthatione S-transferase M (GSTM), which were down-regulated. SOD1, GSTP1 and HSPB1 showed significantly higher content post-RT than Pre-ULLS; CAH III and α-β-crystallin showed significantly higher content Post-RT than Post-ULLS recovering to control values. Western blot analysis confirmed the adaptations in SOD1, PRDX3, α-β-crystallin and HSP1 shown by proteomic analysis (Fig. 7). Moreover, given its relevance among the antioxidant defence systems, catalase, which is not identified by proteomic, was studied by western blotting. Catalase was up-regulated Post-ULLS and returned towards Pre-ULLS levels following rehabilitation.

Several proteins, which could not be ascribed to a specific functional group went also through significant adaptations (Fig.6B). Four and half LIM domain (FHL1) was significantly down-regulated in Post-ULLS compared to Pre-ULLS group. After training an increase in FHL1, EF-Tu and a decrease in proteasome α-sub isoform-2 and in DJ-1 protein was found.

Post-translational modifications

The adaptations in antioxidant defence systems suggested that ULLS and subsequent RT could affect redox balance. To further evaluate redox state, we performed immunoblot detection (Oxyblot) of carbonyl groups introduced into proteins by oxidative reactions. This analysis was carried out separately on both total protein content and myosin heavy chain (MHC) isoforms (Fig.8). The results showed no variations in total protein oxidation index after suspension and recovery. The oxidation of myosin was significantly lower Post-ULLS and Post-RT than Pre-

ULLS. No significant difference in myosin oxidation was observed Post-RT compared to Post-ULLS although a trend towards an increase was observed.

In order to clarify the variations observed in shortening velocity (Vo), not only myosin light chain (MLC) composition (Fig.2B), but also MLC phosphorylation were investigated. No variations were observed also in the phosphorylation degree of MLC-1, MLC-2s and MLC-2f (Fig.8C).

Intracellular signalling pathway analysis

In order to understand the mechanisms underlying changes in fibre size and in protein expression, the major intracellular pathway potentially involved were studied in each subject group. We analysed some master controllers of: pathways controlling muscle mass, namely MuRF1 and Atrogin1 ubiquitin ligases (ubiquitin proteasome system), Beclin1 and p62 (autophagy system), and Akt, S6Rp and 4EPB1 (IGF-1/Akt/mTOR pathway); muscle metabolism, namely PGC-1alpha and SREBP-1; energy balance (AMPK); antioxidant defence systems (NRF2).

No changes were found for MuRF1 and Atrogin1 mRNA level in all conditions. Post-RT, we reported a trend for a decreased expression in both gene levels, but the variation did not reach statistical significance (Fig.9A). Beclin1 expression showed no significant differences in all conditions, while p62 mRNA level post-RT was significantly lower compared to both Pre-ULLS and Post-ULLS group (Fig.9B). Concerning protein synthesis, phosphorylation level of AKT, 4EBP1 and S6 was significantly reduced after disuse and then returned at Pre-ULLS values after exercise, except for AKT that showed low levels of phosphorylation even after exercise (Fig.9C).

AMPK activation and mRNA expression of PGC1α and SREBP1 were analysed (Fig.10). The phosphorylation level of AMPK and the expression of SREBP1 were significantly down-regulated Post-ULLS compared to Pre-ULLS and significantly up-regulated Post-RT compared to Post-ULLS. No differences were found in PGC1α mRNA expression.

Finally, the regulation of antioxidant defence systems was assessed analysing Nuclear factor (erythroid-derived 2)-like 2 (NRF2), a sensor of redox balance and the major transcription nuclear factor involved in modifying antioxidants expression. The NRF2 mRNA level showed no significantly changes after suspension and after recovery (Fig.10C).

Discussion

Qualitative changes in muscle function following disuse and re-training: **force and velocity of shortening of identified types of muscle fibres**

In this study functional properties of single skinned muscle fibres from vastus lateralis muscle of young male subjected to ULLS and active recovery were studied. Maximally activated skinned muscle fibres enable direct analysis of the adaptation of the contractile apparatus itself as excitation-contraction coupling is missing (Bottinelli *et al.*, 1996; Widrick *et al.*, 1997). As expected, ULLS was able to induce significant atrophy (Fig.1) in agreement with previous studies on whole muscle and muscle fibres following several models of disuse in humans (Fitts *et al.*, 2001; Adams *et al.*, 2003; di Prampero & Narici, 2003). The post training recovery of CSA (Fig.1) is expected as it is well known that resistance training induces skeletal muscle fibres hypertrophy (Jones *et al.*, 1989; Green *et al.*, 1999; Widrick *et al.*, 2002a; D'Antona *et al.*, 2006), increases protein translation (Bodine, 2006; Coffey & Hawley, 2007) and enhances expression of genes involved in anabolic mechanisms (Bickel *et al.*, 2005; Kosek *et al.*, 2006; Kvorning *et al.*, 2007).

The difference in the relative loss of CSA of muscle fibres (22-23%) and of vastus lateralis volume (12%) Post-ULLS observed in the same subjects (Campbell *et al.*, 2013) is not surprising as the impact of muscle fibres CSA on muscle volume is complex depending on pennation angle and intramuscular fat and connective tissue content, both of which change with inactivity. Chronic inactivity has been shown to lead to an increase in intramuscular fat (Manini *et al.*, 2007)which may have partially masked the actual decrease in muscle tissue volume. The same reasoning could explain the full recovery of VL volume compared with lack of complete recovery in CSA of muscle fibres (Campbell *et al.*, 2013).

<u>*Specific force*</u> The lower P_0/CSA of muscle fibres Post ULLS (Fig.1) is expected based on several previous observations (Larsson *et al.*, 1996; Widrick *et al.*, 1999b; Widrick *et al.*, 2001; Canepari *et al.*, 2010) although it has not been seen in all disuse studies in humans, among which bed rest (Widrick *et al.*, 1997) and spaceflight studies (Fitts *et al.*, 2010). It is also broadly consistent with a previous muscle fibre analysis following ULLS (Widrick *et al.*, 2002b). The recovery of Po/CSA post-RT is unlikely a consequence of a simple impact of RT on single muscle fibre properties. In fact, no impact of short term RT training on Po/CSA of individual muscle fibres has been shown so far in healthy young subjects (Widrick *et al.*, 2002a; Shoepe *et al.*, 2003; Malisoux *et al.*, 2006; Claflin *et al.*, 2011) notwithstanding an increase in CSA following training (Claflin *et al.*, 2011). It appears, therefore, that the impact of RT on Po/CSA (Fig.1) is linked to the previous loss, which

occurred during ULLS. Indeed, the extent of the gain in force following RT was rather impressive suggesting that muscle fibres, which went through disuse atrophy are very responsive to resistance exercise (Trappe, 2009). The latter phenomenon could be due to the phenomenon of 'muscle memory' which describes a faster recovery of muscle mass post inactivity due to preservation of myonuclei during the unloading period (Bruusgaard *et al.,* 2012). Although a disproportionate loss of force compared to CSA in individual muscle fibres can be considered a major determinant of the lower specific force of whole muscles in vivo following disuse (Dudley *et al.*, 1992; Adams *et al.*, 1994; Schulze *et al.*, 2002; di Prampero & Narici, 2003; Narici *et al.*, 2003), a direct demonstration could not be provided as in vivo and in vitro analyses were performed in different subjects. In this study, analysis of muscle fibre Po/CSA was performed on muscle samples from the same experiment and subjects in which we determined structure and function of quadriceps muscles in vivo (Campbell *et al.,* 2013). Collectively the results indicate that the loss of Po/CSA at muscle fibre level (22-23%) can play a relevant role in the decrease in specific force (19-24%) of the quadriceps muscles in vivo. Importantly, in fact, in vivo physiological cross sectional area, and maximal voluntary activation and neural drive, which could also contribute to the phenomenon, did not change following ULLS in this specific experiment (Campbell *et al.*, 2013).

In this study, myosin content was determined in the same muscle fibres used for Po/CSA determination in young subjects. Importantly, the parallel changes observed in Po/CSA and myosin concentration in both type 1 and 2A fibres following disuse and rehabilitation strongly support the role of changes in myosin content in accounting for the changes in Po/CSA in disuse and recovery. However, notwithstanding the linear relation previously observed between Po/CSA and myosin concentration by D'Antona et al. (D'Antona *et al.*, 2003) and the parallel changes in Po/CSA and myosin concentration observed in this study, it is still unsettled whether changes in myosin concentration can account for the all adaptations in Po/CSA. Indeed, a loss of Po/CSA could depend not only on a decrease in the number of myosin heads in the CSA, but also on lower force generated per myosin head or on a lower fraction of myosin heads interacting with actin at any given time. Interestingly, myosin content post-RT was the same as Pre-ULLS (Fig.2), whereas Po/CSA post-RT recovered above Pre-ULLS levels (Fig.1). It has been recently suggested that disuse in elderly subjects can alter acto-myosin kinetics, which in turn affect Po/CSA regardless changes in myosin quantity (Callahan *et al.*, 2014).

Unloaded shortening velocity Vo has been shown to be higher, lower, or unchanged following disuse in humans, i.e. the adaptations of Vo varied according to models of disuse, subjects groups, muscles and fibre types (Widrick *et al.*, 1999b) (Larsson *et al.*, 1996; Widrick *et al.*, 2001; Trappe

et al., 2004). Following ULLS, Vo was lower in type 1 fibres from soleus and higher in type 2A fibres from gastrocnemius, whereas no changes were observed in type 2A fibres from soleus and type 1 and 2X fibres from gastrocnemius (Widrick *et al.*, 2002b). In the present study, ULLS did not induce changes in shortening velocity in both fibre types (Fig.1). Interestingly, following rehabilitation, Vo of both type 1 and 2A fibres increased above Pre-ULLS values (Fig.1). The impact of resistance training on V_0 in young subjects has not been clearly established yet. In most training studies no change in V_0 was observed (Harridge *et al.*, 1998; Widrick *et al.*, 2002a). Conversely, young women showed higher V_0 in both type 1 and type 2A fibres after 1 year of resistance training (Pansarasa *et al.*, 2009) and highly trained male swimmers had 32% and 67% higher Vo in slow and fast fibres respectively after tapering (Trappe *et al.*, 2000).

In an attempt to clarify the mechanisms underlying the higher Vo of muscle fibres post-RT than Pre-ULLS several hypothesis were forwarded (Bottinelli & Reggiani, 2000; Canepari *et al.*, 2010) and verified: changes in MLC isoform content; changes in regulatory MLC phosphorylation; post-translational modification of myosin. Vo has been shown to be modulated by the MLC3f/MLC2f ratio (Bottinelli *et al.*, 1994) and to be possibly affected by the MLC2s and MLC2f content in human fibres (Bottinelli & Reggiani, 2000). Moreover, regulatory light chain phosphorylation-induced decreased velocity was observed in single muscle fibres and in isolated myosin in vitro motility assay (Diffee *et al.*, 1996; Olsson *et al.*, 2004; Greenberg *et al.*, 2009). No differences were found either in MLC content (Fig.2) or in phosphorylation (Fig.8) among Pre-ULLS, Post-ULLS and Post-RT muscle samples.

Myosin oxidation has been shown to decrease actin-sliding velocity in in vitro motility assays (Coirault *et al.*, 2007; Maffei *et al.*, 2014). It could be hypothesized that RT determined higher Vo causing a decrease in myosin oxidation. However, myosin was less oxidized post-ULLS and slightly more post-RT (Fig.8). It is therefore very unlikely that MLC composition or phosphorylation, and myosin oxidation can account for the higher Vo post-RT. However, a potential role of a change in the properties of myosin itself in causing the latter phenomenon can still be hypothesised based on the observation that RT increased actin-sliding velocity on pure myosin isoforms in in vitro motility assays in both in young and OLD subjects (Canepari *et al.*, 2005).

Qualitative changes following disuse and re-training: myosin and myofibrillar proteins

Myosin Heavy Chain (MHC) composition. Although a slow to fast shift in MHC isoform distribution is generally believed to occur in disuse, such phenomenon has not been seen in all conditions of decreased neuromuscular activity (di Prampero & Narici, 2003). The lack of any

significant MHC isoform shift following three weeks ULLS (Fig.2) is, therefore, consistent with previous results.

Thin filament proteins The proteomic analysis showed two major phenomena at the level of myofibrillar proteins: a down regulation of several thin filament spots protein and no shift from slow to fast isoforms. The large changes in Tn spots abundance, as determined by 2D gels, would suggest a major loss of the thin filament. It should be noted that, since we have not been able to confirm the levels of Tn spots expression through the immunoblotting, it is possible that the large downregulation of TnIf and TnTf spots after disuse could not reflect the actual expression levels of troponin. In agreement with this, the assessment of myosin actin ratio has suggested a greater loss of myosin content than actin (Fig.2D). The down-regulation of thin filament proteins, is consistent with the lower myosin content and indicate a disproportionate loss of myofibrils compared to the remainder muscle proteins in the atrophic muscle fibres. The latter observation is not in agreement with an analysis of myosin and myofibrillar content following 35 days of ULLS (Carrithers *et al.*, 2002; Haus *et al.*, 2007), but is broadly consistent with several previous findings: the analysis of total mRNA and mRNA for actin and myosin heavy chain isoforms has indicated a preferential decrease in myosin and actin transcription within an overall decrease in muscle protein transcription following 5 weeks of ULLS (Haddad *et al.*, 2005); the analysis of thin filament density has confirmed a loss of actin (Riley *et al.*, 2002) following 17 days of spaceflight.

Among the down-regulated myofibrillar proteins, myozenin-1 is particularly interesting. It binds Z-disk proteins, α-actinin and γ-filamin affecting dimerization of these proteins and modulating the spacing between monomers and hence the spacing between actin-thin filaments (Thompson *et al.*, 2000; van der Ven *et al.*, 2000). Interestingly, the density of thin filaments has been seen to decrease following disuse (Riley *et al.*, 2000).

The observation that 3 weeks of RT mostly counteracted the down-regulation of myofibrillar proteins following 3 weeks of ULLS indicates that skeletal muscle following disuse can still respond to training and points out the relevance of rehabilitation.

Qualitative changes following disuse and re-training: metabolic enzymes

A global down-regulation of glycolytic enzymes and other metabolic systems like creatine kinase (CK) and adenylate kinase (AK) was found after lower limb suspension by proteomic analysis (Fig.5). The down-regulation of two oxidative enzymes, NADH dehydrogenase Fe-S and ATP-synthase, Post-ULLS did not reach statistical significance.

CK plays an important role in high energy phosphoryl transfer and cellular energy buffering (Bessman & Carpenter, 1985; Wallimann *et al.*, 1992; van Deursen *et al.*, 1993) and AK is

implicated in cellular adenine nucleotide homeostasis (Walker-Simmons & Atkinson, 1977). CK and AK, in concert with enzymes which function in glycolytic phosphotransfer pathway, form the cellular energetic infrastructure responsible for handling and distribution of high energy phosphoryl groups throughout the structured muscle environment (Bessman & Carpenter, 1985; van Deursen *et al.*, 1993; Janssen *et al.*, 2000; Abraham *et al.*, 2002). Altogether, our data indicate a general impairment in energy production after three weeks of ULLS.

Resistance training has been shown to enhance glycolytic metabolism (Holloway *et al.*, 2009; Friedmann-Bette *et al.*, 2010). Consistently glycolytic enzymes, which were down-regulated following disuse, were up-regulated post-RT and their content was mostly restored to Pre-ULLS levels (Fig.5). RT determined also an increase in two oxidative enzymes (NADH dehydrogenase Fe-S and ATP-synthase) (Fig.5), consistently with what observed by proteomic analysis following 6 weeks of RT (Holloway *et al.*, 2009). Therefore, although CK content expression was unaffected by RT, it appears that RT was able to counteract the derangement of energy metabolism observed following ULLS restoring a normal energy metabolism. The up-regulation of oxidative enzymes is unlikely to be attributed to PGC-1alpha as only a trend towards its increased expression was observed (Fig.10).

Response of intracellular signalling pathways controlling muscle mass and metabolism following disuse and re-training

Pathways controlling muscle mass Muscle fibre atrophy and loss of myosin, myofibrillar proteins and metabolic enzymes following ULLS prompted the analysis of the ubiquitin-proteasome and autophagy systems which are the two major, ATP-dependent intracellular signalling pathways controlling muscle protein breakdown and muscle protein synthesis (Sandri, 2008).

MuRF1 and atrogin1 are key ubiquitin ligases and are the atrogenes most widely used as markers of the ubiquitin-proteasome activity (Sandri *et al.*, 2004; Brault *et al.*, 2010; Romanello *et al.*, 2010). The unchanged expression of MuRF1 and atrogin1 after 3 weeks of disuse (Fig.9) is not surprising and is consistent with several previous observations in humans (de Boer *et al.*, 2007; Alibegovic *et al.*, 2010). It is has become increasingly clearer, in fact, that atrogenes activation occurs transiently and at early times in disuse atrophy of both human (de Boer *et al.*, 2007; Urso *et al.*, 2007; Sakuma *et al.*, 2009; Gustafsson *et al.*, 2010) and mice (Cannavino *et al.*, 2014) limb muscles. Collectively, the present findings do not support a significant contribution of the ubiquitin-proteasome system in the progression of muscle atrophy at late stages of ULLS, but do not rule out the possibility that the early developing of muscle atrophy depended on such system. The conclusion is consistent with what observed at late stages of bed rest (Brocca *et al.*, 2012).

The autophagy system is constitutively active in skeletal muscle to remove protein aggregates and old or damaged organelles (Sandri, 2010). Moreover, it has been recently understood that autophagy system can play a relevant role in protein degradation and in muscle atrophy (Sandri, 2010). In a recent study, an up-regulation of autophagy has been suggested to contribute to the progression of muscle atrophy at late stages (23 days) of bed rest (Brocca *et al.*, 2012). On the contrary, the unchanged expression of Beclin-1 and p62 (Fig. 9B) does not support a role of autophagy in ULLS.

Several evidences have suggested a major role of decreased protein synthesis in causing disuse atrophy in humans (de Boer *et al.*, 2007; Glover *et al.*, 2010; Rennie *et al.*, 2010). Among the different pathways controlling muscle size (Glass, 2003, 2005, 2010; Lee & Glass, 2011) the IGF-1/Akt/mTOR pathway is central and is mainly recruited upon IGF-1/insulin stimuli to promote protein synthesis and to block protein degradation (Rommel *et al.*, 2001). After unloading we found changes in the phosphorylation level of Akt, 4EBP1 and S6 suggesting that a decline in protein synthesis could contribute to the progression of muscle atrophy at late stages of ULLS, in agreement with previous observations (de Boer *et al.*, 2007; Glover *et al.*, 2010; Rennie *et al.*, 2010). Interestingly, at late stages of bed rest, no significant changes in the IGF-1/Akt/mTOR pathway were observed (Brocca *et al.*, 2012).

It is well known that resistance training causes muscle hypertrophy and enhanced rates of protein synthesis (Murton *et al.*, 2008) (Rennie & Tipton, 2000). After exercise a transient increase in phosphorylation of AKT, mTOR, PKB, 4EBP1 and p70/S6K was, in fact, reported (Baar & Esser, 1999; Nader & Esser, 2001; Bolster *et al.*, 2003; Parkington *et al.*, 2003; Kubica *et al.*, 2005; Cuthbertson *et al.*, 2006). Consistently, we show an increase of phosphorylation of 4EBP1 and S6K; no changes were found for AKT (Fig.9).

It is still unclear whether the activity of the ubiquitin proteasome system is enhanced (Yang *et al.*, 2006; Louis *et al.*, 2007; Raue *et al.*, 2007; Deldicque *et al.*, 2008; Mascher *et al.*, 2008) or inhibited (Baar *et al.*, 2006) by resistance exercise. Furthermore, recent works illustrated a down-regulation of several markers of autophagy (like LC3 and GABARAP) following resistance training in human (Glynn *et al.*, 2010; Fry *et al.*, 2012) suggesting a decrease in autophagy flux. The trend towards a down-regulation of MuRF1, atrogin1 and the reduction of p62 mRNA (Fig. 8) in Post-RT group suggest that muscle mass recovery following ULLS could be due to both an enhanced protein synthesis and a decreased protein degradation.

Pathways controlling muscle metabolism PCG-1alpha is a master controller of mitochondrial biogenesis and oxidative metabolism (Lin *et al.*, 2002). Moreover, as it inhibits the FoxO proteolytic pathway, lower PGC-1α expression can be associated with muscle wasting (Sandri *et*

al., 2006; Sacheck *et al.*, 2007) and occurred after disuse in human (Brocca *et al.*, 2012) and in mice (Cannavino *et al.*, 2014). In human bed rest, PGC-1alpha down-regulation has been suggested to be a major determinant of the disuse induced metabolic derangement, whereas no clear role in causing muscle atrophy has been established (Brocca *et al.*, 2012). In mice, PCG-1alpha down-regulation following hind-limb unloading has been shown to play a major role not only in metabolic derangement, but also in muscle mass loss (Cannavino *et al.*, 2014).

In the present study, the lack of PCG-1alpha down-regulation suggests that, on the contrary of what happens in human bed rest (Brocca *et al.*, 2012) and in mice hindlimb unloading (Cannavino *et al.*, 2014), PCG-1alpha does not play a significant role in muscle adaptations following 3 weeks of ULLS, i.e. at least in the late stages of disuse. The observation is consistent with the lack of a significant impairment of oxidative metabolism (Fig.5). Exercise training is known to enhance PGC-1alpha expression (Pilegaard *et al.*, 2003). The lack of effect of resistance training program on PGC-1α expression suggests that disuse blunts PGC-1alpha response to exercise consistently with previous observations (Ringholm *et al.*, 2011). It should be noted that, notwithstanding the resistance to up-regulation of PGC-1alpha, resistance exercise was able to counteract the negative effects induced by unilateral lower leg suspension on energy production systems.

SREBPs are master regulators of lipid homeostasis, inducing transcription of many genes involved in lipid biosynthesis (Horton *et al.*, 2002). SREBP-1 stimulates lipogenesis and triglyceride deposition and plays an important role in regulating lipid metabolism in skeletal muscle (Horton *et al.*, 2002; Commerford *et al.*, 2004). An increase in SREBP-1 expression was observed following 23 days of bed rest and was suggested to be involved in intramuscular accumulation of lipids and in impaired mitochondrial function (Brocca *et al.*, 2012). The lower expression of SREBP-1 following ULLS suggest a differential response of lipid metabolism of human skeletal muscle to ULLS and bed rest. The up-regulation of SREBP-1 post-RT counterbalances the down-regulation observed in disuse and suggests an exercise-induced modulation of lipid metabolism and an enhancement of lipid accumulation.

The AMPK system senses the energy level of the cell, i.e. the ratio between AMP and ATP, and counteracts energy imbalance increasing protein break-down through FoxO activation and decreasing protein synthesis through inhibition of IGF-1/Akt/mTOR pathway. We have previously shown no changes of the AMPK system following 23 days of bed rest, suggesting that no energy stress occurred late in bed rest, notwithstanding an impairment of energy metabolism (Brocca *et al.*, 2012). Differently from bed rest model, here we found low levels of activation of AMPK pathway after disuse (Fig.10). The same was observed in the vastus lateralis of individuals with extreme

muscular disuse, indicating a deregulated fatty acid oxidation (Kostovski *et al.*, 2013). After exercise, the level of AMPK activation returned to the control value in accordance with the known role that the muscular contraction exerts on AMPK activation (Nielsen *et al.*, 2003).

Redox balance following disuse and re-training

Oxidative stress is widely believed to be a major trigger of muscle adaptations to disuse. Whereas its role has been elegantly defined in the dramatic (50%) and fast developing (12-18 hours) diaphragm atrophy following mechanical ventilation (Levine *et al.*, 2008), in human limb muscles analyses of redox status are few and contradictory results have been reported (Pellegrino *et al.*, 2011b). After 35 days of bed rest an impairment of antioxidant defence system and increased protein carbonylation was found in vastus lateralis muscle (Dalla Libera *et al.*, 2009; Brocca *et al.*, 2012). Moreover, a recent analysis of gene expression (Reich *et al.*, 2010) has shown an up-regulation of the pathways involved in oxidative stress following 48h of unilateral lower leg suspension. The concomitant increase of SOD1, Catalase and PRDX3 Post-ULLS (Fig.6) could be part of successful compensatory mechanisms against increased ROS production. The latter hypothesis is supported by the absence of protein carbonylation (Fig.8) and by unchanged expression of NRF2 (Fig.10), one of the most important transcription factors responsible for expression of detoxification enzymes, that is indirectly activated by ROS over-production. Muscular activity promotes ROS production in contracting skeletal muscle fibres from a variety of cellular locations (Powers *et al.*, 2009). Early studies focused on the damaging effects of ROS, but more recent studies have shown that ROS production is necessary to enable muscle adaptations to exercise (Powers & Jackson, 2008). Moreover, physiological levels of ROS are essential for optimal force production in skeletal muscle, but high levels of ROS promote skeletal muscle contractile dysfunction resulting in muscle fatigue (Powers *et al.*, 2007).

Following exercise the imbalance between ROS levels and antioxidant systems can cause oxidative protein modifications including protein carbonylation (Barreiro & Hussain, 2010). Consistently with an alteration of redox balance Post-RT, SOD 1, CAHIII and two heat shock protein (HSPB1, α-β-crystallin) were up-regulated and PRDX3 and catalase were down-regulated compared to Post-ULLS (Fig.6). The up-regulation of heat shock proteins post-RT is consistent with previous findings (Fujino *et al.*, 2009) (Powers *et al.*, 2009) (McArdle & Jackson, 2000). It could be involved in the localization of newly synthesized proteins following exercise and could provide cytoprotection against subsequent stresses. The latter findings suggest that human skeletal muscle following long term disuse can still effectively respond to enhanced ROS production due to

exercise. The latter hypothesis is supported by the lack of up-regulation of NRF2 (Fig.10) and of protein carbonylation Post-RT (Fig. 8).

Conclusions

Our findings support the hypotheses that (i) specific force of muscle fibres decreased following ULLS and went back to normal following three weeks active recovery due to a loss and recovery of myosin and actin content and (ii) profound alterations of muscle protein pattern occurred during ULLS and were reversed during recovery, but do not support the hypothesis that (iii) oxidative stress and protein carbonylation occurred during ULLS and redox balance was restored by active recovery. Indeed, adaptations of antioxidant defence systems were observed following ULLS, and were reversed by active recovery, but no sign of oxidative stress and protein carbonylation were found in either conditions suggesting an effective cellular response to redox imbalance. The latter observations do not support a major role of oxidative stress in ULLS.

Muscle atrophy following ULLS could not be related to activation of pathways causing muscle protein breakdown (ubiquitin-proteasome and autophagy systems), whereas a down-regulation of protein synthesis could be hypothesized.

Resistance training was very effective in restoring both muscle atrophy and qualitative muscle changes indicating that long term ULLS did not prevent the positive effect of exercise on human muscle.

Interestingly, the response to 3 weeks ULLS showed similarities and differences compared to the response to 23 days bed rest (Borina *et al.*, 2010; Brocca *et al.*, 2012). The loss in muscle fibres CSA (23%) and myosin concentration (28%) was very similar. In both conditions, profound qualitative adaptations occurred both in the pattern of myofibrillar proteins and metabolic enzymes. In both conditions, adaptations of antioxidant defence systems was observed. However, whereas in bed rest all defence systems were down-regulated, NRF2 was consistently up-regulated and protein carbonylation occurred indicating that oxidative stress developed, in ULLS antioxidant defence systems were up-regulated, NRF2 was unchanged and no protein carbonylation occurred indicating an effective cellular response to redox imbalance. Moreover, whereas in bed rest autophagy was up-regulated potentially contributing to atrophy progression and PGC-1alpha was down-regulated supporting metabolic derangement, in ULLS no autophagy activation and no PGC-alpha down-regulation occurred.

Competing interests

The authors declare no conflict of interest.

Author contributions

R.B., M.A.P. and L.B.: conception and design of the experiments; L.B., E.L., J.C., O.S., J.MP: collection, analysis and interpretation of data; GdV: collection of biopsies and supervision of the in vivo experiment; L.B., M.A.P., M.N. and R.B.: drafting the article or revising it critically for important intellectual content.

All authors made comments on the manuscript and read and approved the final version.

The biopsies were performed at Institute for Biomedical Research into Human Movement and Health, Manchester Metropolitan University, Manchester, United Kingdom; all the other experiments were performed at the Department of Molecular Medicine, University of Pavia, Italy.

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Figure 1: Structure and function of single muscle fibers from vastus lateralis are affected by disuse and active recovery.

A. cross-sectional area (CSA), *B.* specific force (P_0/CSA), *C.* maximum shortening velocity (V_0) of type-1 and type-2A fibers dissected from vastus lateralis muscles of Pre-ULLS, Post-ULLS and Post-RT groups. CSA is expressed in μm^2, P_0/CSA is expressed in kNm^{-2} and V_0 is expressed in LS^{-1}.

• = significantly different from Pre-ULLS ($P < 0.05$); ★ = significantly different from Post-ULLS ($P < 0.05$). Data are presented as mean values ± SEM.

Figure 2: Myosin concentration and myosin/actin ratio decreased after three weeks of disuse and returned to control values after active recovery.

A. Myosin heavy chain (MHC) isoforms composition, *B.* myosin light chain (MLC) isoforms composition, *C.* myosin concentration in type-1 and type-2A fibers dissected from vastus lateralis muscles and *D.* myosin/actin ratio in Pre-ULLS, Post-ULLS and Post-RT groups.

• = significantly different from ULLS-Pre ($P < 0.05$); ★ = significantly different from ULLS-Post ($P < 0.05$).

Data are presented as mean values ± SEM.

Figure 3: Representative two-dimensional gels of vastus lateralis muscle.

Thirteen centimeter IPG gel strips, pH 3–11 NL (non-linear), were used in the first dimension, and SDS gels (15% T, 2.5% C) were used in the second dimension. The protein spots, which were found to be differentially expressed, are circled and numbered. The numbers enable to identify the spots in Table 1.

Figure 4: Myofibrillar proteins content decreased after three weeks of muscle disuse and partially recovered after exercise.

Histogram representing the volume of 2DE spots of differentially expressed myofibrillar proteins in Pre-ULLS, Post-ULLS and Post-RT groups. • = significantly different from ULLS-Pre ($P < 0.05$); ★ = significantly different from ULLS-Post ($P < 0.05$). Data are presented as mean values ± SEM.

The full information on the proteins and the numerical ratios between volumes are reported Table 1.

Figure 5: Metabolic proteins expression decreased after three weeks of muscle disuse and partially recovered after exercise.

Histogram representing the volume of 2DE spots of differentially expressed metabolic proteins. *A.* Glycolytic proteins, *B.* oxidative proteins, creatin kinase and adenilate kinase in Pre-ULLS, Post-ULLS and Post-RT groups. • = significantly different from ULLS-Pre ($P < 0.05$); ★ = significantly different from ULLS-Post ($P < 0.05$). Data are presented as mean values ± SEM.

The full information on the proteins and the numerical ratios between volumes are reported in Table 1.

Figure 6: Antioxidant defense systems content efficiently adapted to ROS production induced by disuse and resistance training.

Histogram representing the volume of 2de spots of differentially expressed proteins of *A.* antioxidant defense systems and *B.* Other proteins in Pre-ULLS, Post-ULLS and Post-RT groups.

• = significantly different from ULLS-Pre ($P < 0.05$); ★ = significantly different from ULLS-Post ($P < 0.05$). Data are presented as mean values ± SEM.

The full information on the proteins and the numerical ratios between volumes are reported in Table 1.

Figure 7: Validation of antioxidant and metabolic protein expression changes by western blot analysis.

Quantification of protein levels by western blot analysis of: *A.* catalase, Cu/Zn Superoxide dismutase, Peroxiredoxin 3, α-β-crystallin, HSPB1; *B.* Triosephosphate isomerase, lactate dehydrogenase, beta-enolase, aldolase in Pre-ULLS, Post-ULLS and Post-RT groups. • = significantly different from ULLS-Pre ($P < 0.05$); ★ = significantly different from ULLS-Post ($P < 0.05$). Data are presented as mean values ± SEM.

Figure 8: Determination of post-translational modifications after disuse and active recovery.

Mean values ± SEM. of total carbonylated protein (A) and MHC carbonylation level (B) assessed by Oxyblot analysis of vastus lateralis in Pre-ULLS, Post-ULLS and Post-RT groups. *C.* example of Oxyblot assay and relative red ponceau stain. *D* mean values ± SEM. of MLC phosphorilation in vastus lateralis in Pre-ULLS, Post-ULLS and Post-RT groups. E example of phosphoprotein stain and relative Coomassie stain.

• = significantly different from ULLS-Pre ($P < 0.05$).

Figure 9: Protein synthesis pathway was down-regulated after muscle disuse and recovered following exercise.

Quantification of mRNA level of two major atrogenes (MuRF1 and atrogin1) (A) and autophagy markers (p62 and Beclin-1)(B). mRNA expression was assessed by quantitative RT-PCR in vastus lateralis of Pre-ULLS, Post-ULLS and Post-RT groups. *C.* determination of phosphorylation levels of AKT, 4EBP1 and S6R and relative representative western blot in vastus lateralis of Pre-ULLS, Post-ULLS and Post-RT groups. Data are presented as mean values ± SEM.

• = significantly different from ULLS-Pre ($P < 0.05$); ★ = significantly different from ULLS-Post ($P < 0.05$).

Figure 10: Disuse and resistance training affected AMPK pathway and lipid metabolism.

A. determination of phosphorylation levels of AMPK and relative representative western blot; *B.* mRNA level of PCG1α, SREBP1 and NRF2 in vastus lateralis of Pre-ULLS, Post-ULLS and Post-RT groups subjects. Data are presented as mean values ± SEM. • = significantly different from ULLS-Pre ($P < 0.05$); ★ = significantly different from ULLS-Post ($P < 0.05$).

Table 1. Differentially expressed proteins in vastus lateralis muscles in Pre-ULLS, Post-ULLS and Post-RT groups.

In the table are showed: the spot number corresponding to the number reported in Fig.3, the protein name, the abbreviation, the accession number corresponding to Expasy, the estimated pI in 2D gel, the estimated Molecular Weight in 2D gel, the MOWSE score.

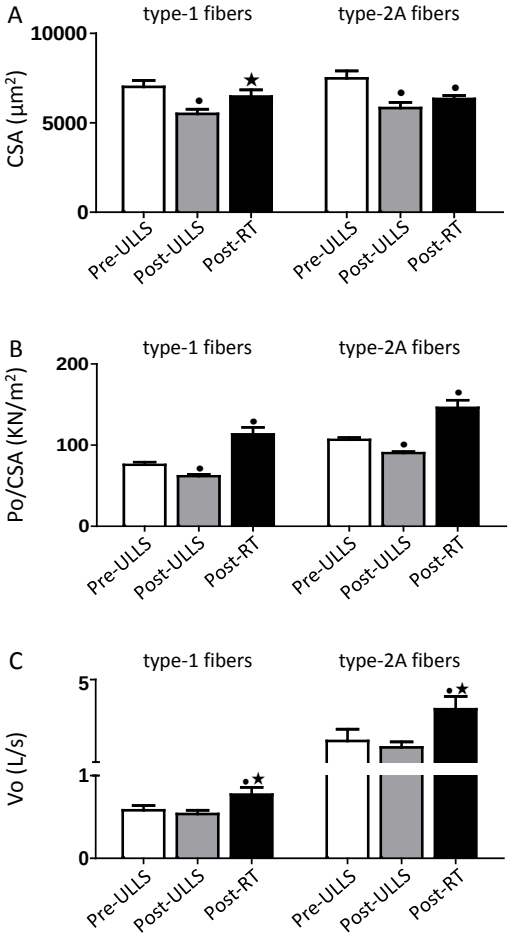
A
type-1 fibers
type-2A fibers
10000
5000
0
CSA (μm²)
Pre-ULLS
Post-ULLS
Post-RT
Pre-ULLS
Post-ULLS
Post-RT
B
type-1 fibers
type-2A fibers
200
100
0
Po/CSA (KN/m²)
Pre-ULLS
Post-ULLS
Post-RT
Pre-ULLS
Post-ULLS
Post-RT
C
type-1 fibers
type-2A fibers
5
1
0
Vo (L/s)
Pre-ULLS
Post-ULLS
Post-RT
Pre-ULLS
Post-ULLS
Post-RT

Fig. 1

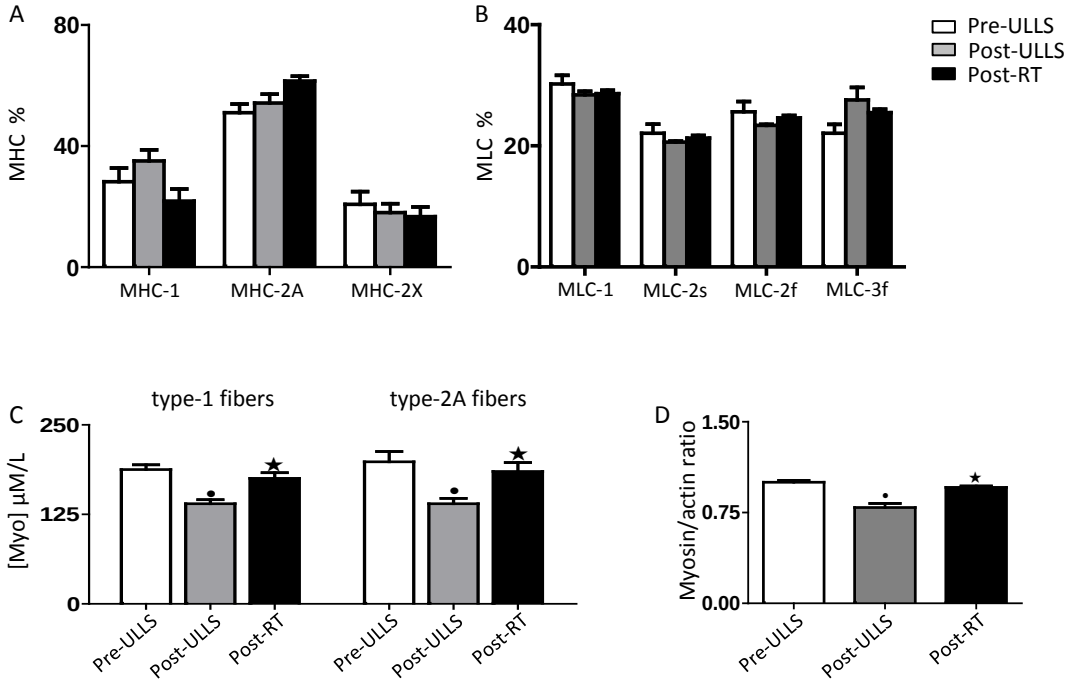
A
MHC %
80
40
0
MHC-1
MHC-2A
MHC-2X
B
MLC %
40
20
0
MLC-1
MLC-2s
MLC-2f
MLC-3f
Pre-ULLS
Post-ULLS
Post-RT
C
type-1 fibers
type-2A fibers
[Myo] µM/L
250
125
0
Pre-ULLS
Post-ULLS
Post-RT
D
Myosin/actin ratio
1.50
0.75
0.00
Pre-ULLS
Post-ULLS
Post-RT

Fig. 2

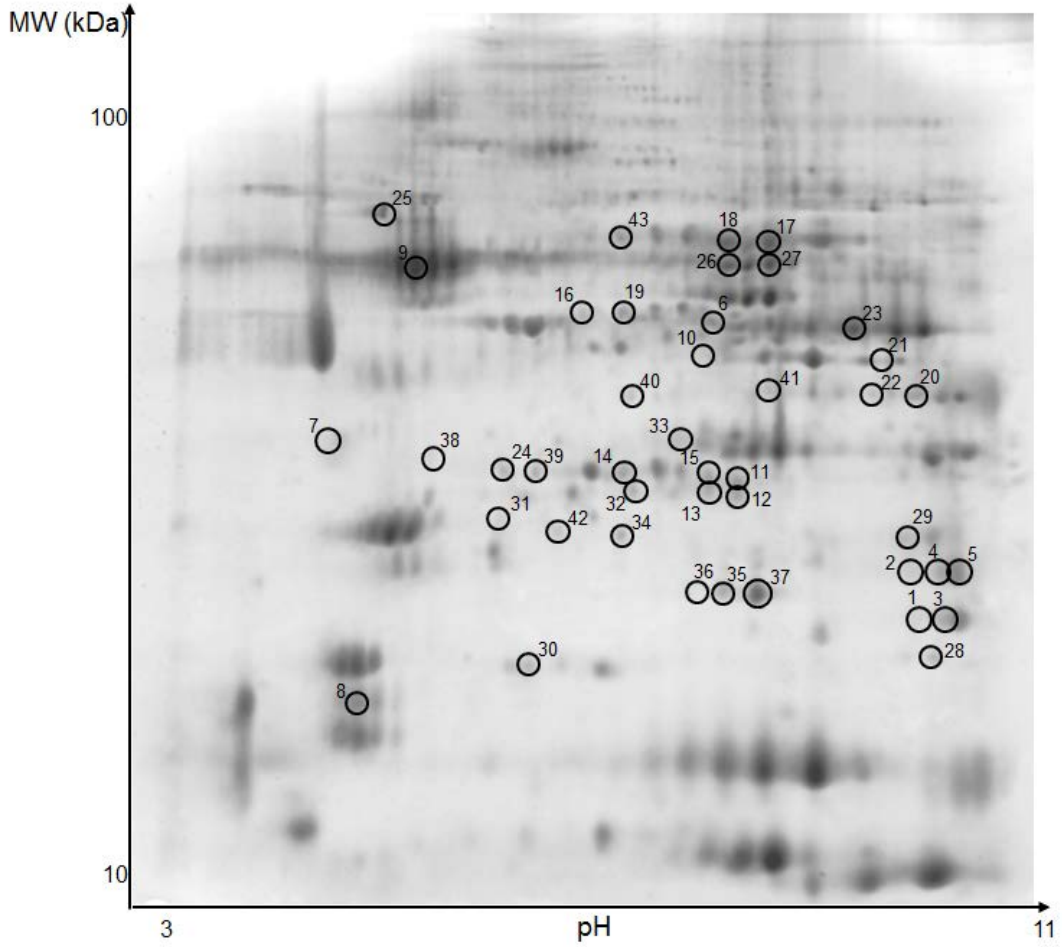
MW (kDa)
100
10
3
pH
11
25
9
43
18
17
26
27
16
19
6
23
10
21
40
41
22
20
7
38
33
24
39
14
15
11
31
32
12
13
42
34
29
4
5
2
36
35
37
1
3
30
28
8

Fig. 3

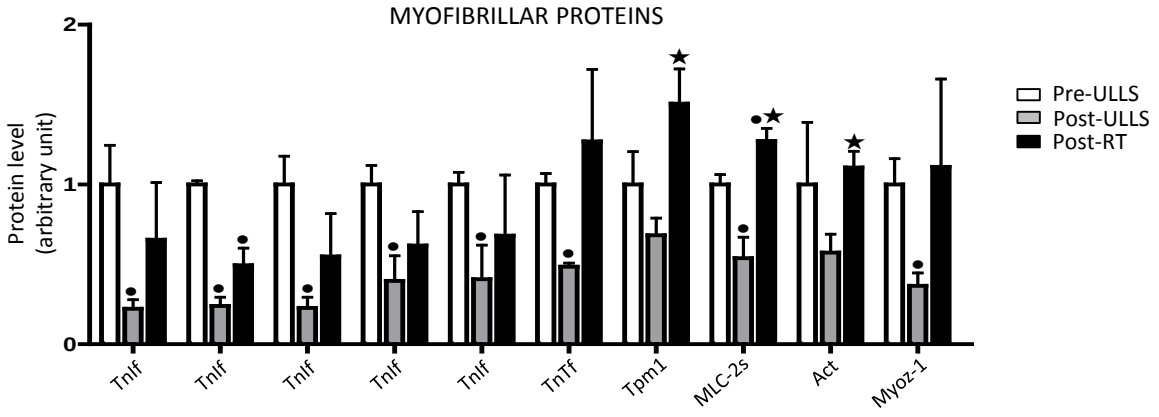
MYOFIBRILLAR PROTEINS
Protein level
(arbitrary unit)
2
1
0
Pre-ULLS
Post-ULLS
Post-RT
Tnlf
Tnlf
Tnlf
Tnlf
Tnlf
TnTf
Tpm1
MLC-2s
Act
Myoz-1

Fig. 4

ENERGY PRODUCTION SYSTEMS

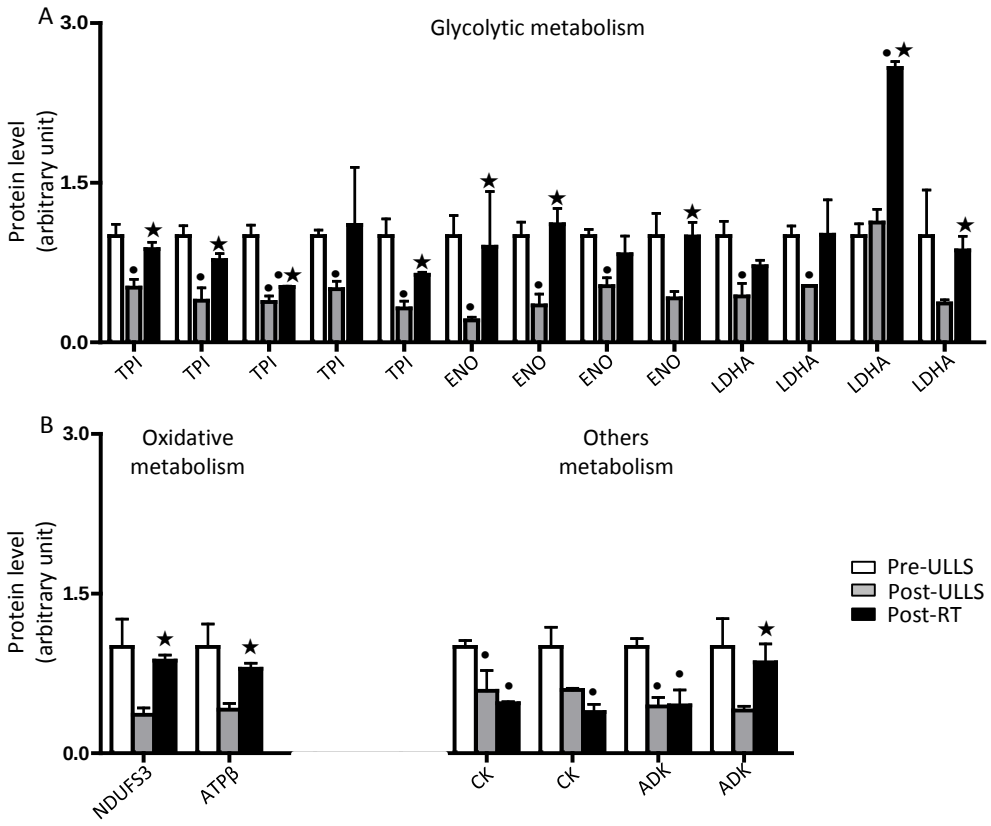

Fig. 5

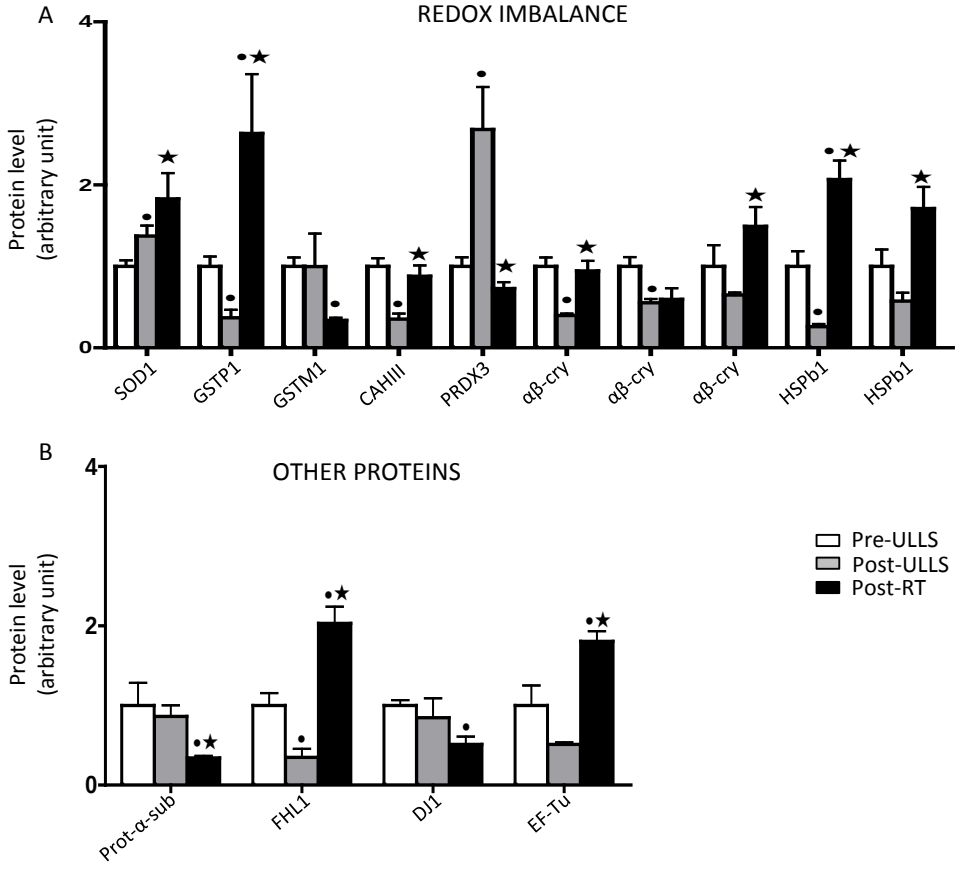
A
REDOX IMBALANCE
Protein level
(arbitrary unit)
4
2
0
SOD1
GSTP1
GSTM1
CAHIII
PRDX3
αβ-cry
αβ-cry
αβ-cry
HSPb1
HSPb1
B
OTHER PROTEINS
Protein level
(arbitrary unit)
4
2
0
Prot-α-sub
FHL1
DJ1
EF-Tu
Pre-ULLS
Post-ULLS
Post-RT

Fig. 6

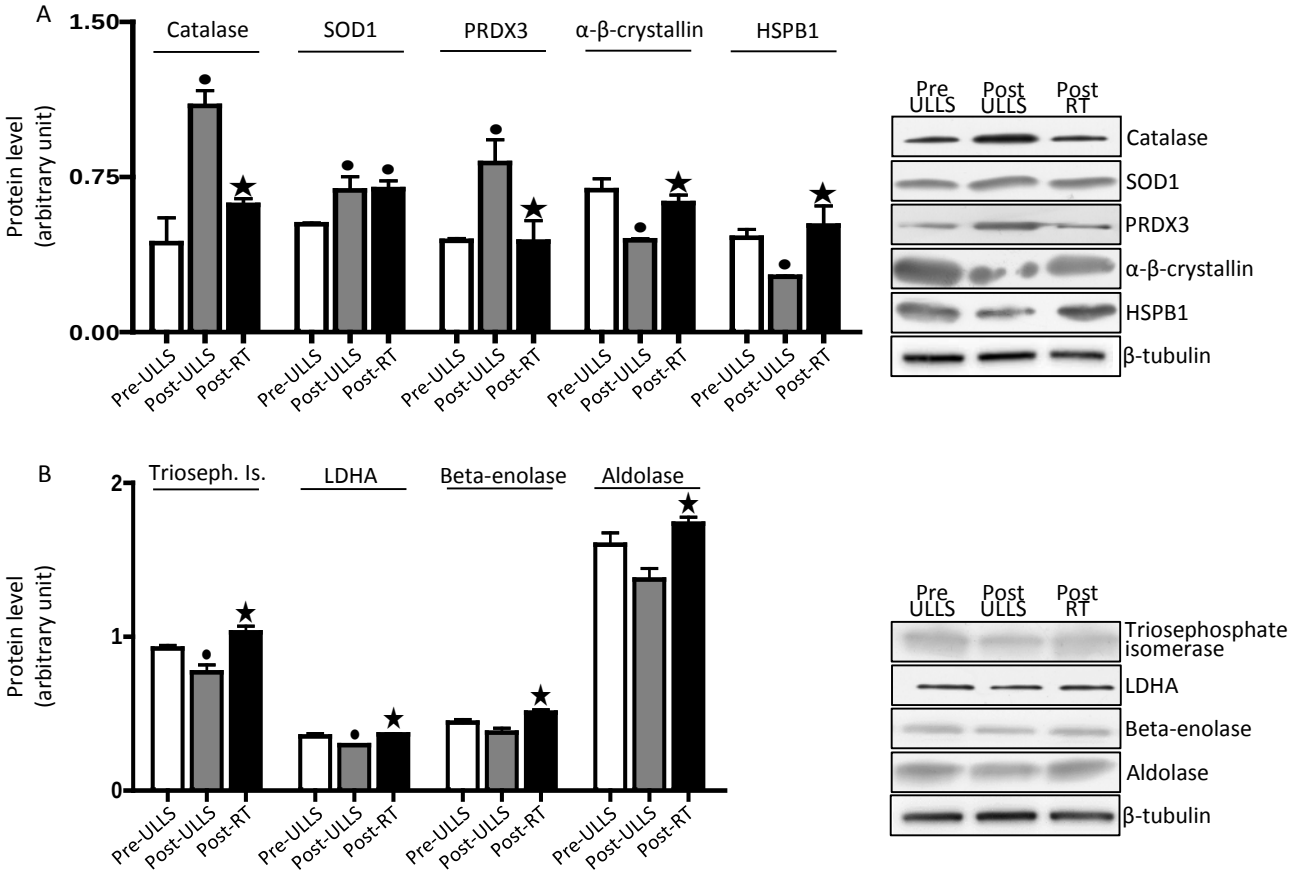
A
1.50
0.75
0.00
Protein level (arbitrary unit)
Catalase
SOD1
PRDX3
α-β-crystallin
HSPB1
Pre-ULLS
Post-ULLS
Post-RT
Pre ULLS
Post ULLS
Post RT
Catalase
SOD1
PRDX3
α-β-crystallin
HSPB1
β-tubulin
B
2
1
0
Protein level (arbitrary unit)
Trioseph. Is.
LDHA
Beta-enolase
Aldolase
Triosephosphate isomerase
β-tubulin

Fig. 7

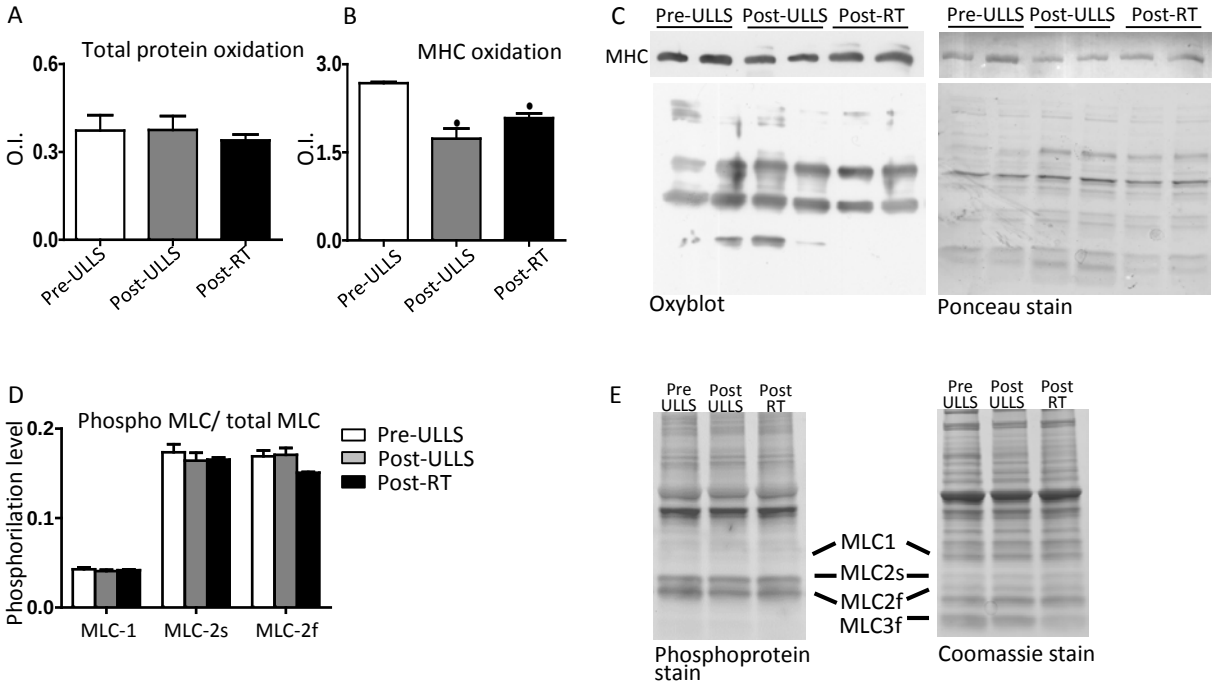
A
Total protein oxidation
O.I.
0.6
0.3
0.0
Pre-ULLS
Post-ULLS
Post-RT
B
MHC oxidation
O.I.
3.0
1.5
0.0
Pre-ULLS
Post-ULLS
Post-RT
C
Pre-ULLS Post-ULLS Post-RT
MHC
Oxyblot
Pre-ULLS Post-ULLS Post-RT
Ponceau stain
D
Phospho MLC/ total MLC
Phosphorilation level
0.2
0.1
0.0
MLC-1
MLC-2s
MLC-2f
Pre-ULLS
Post-ULLS
Post-RT
E
Pre ULLS
Post ULLS
Post RT
MLC1
MLC2s
MLC2f
MLC3f
Phosphoprotein stain
Coomassie stain

Fig. 8

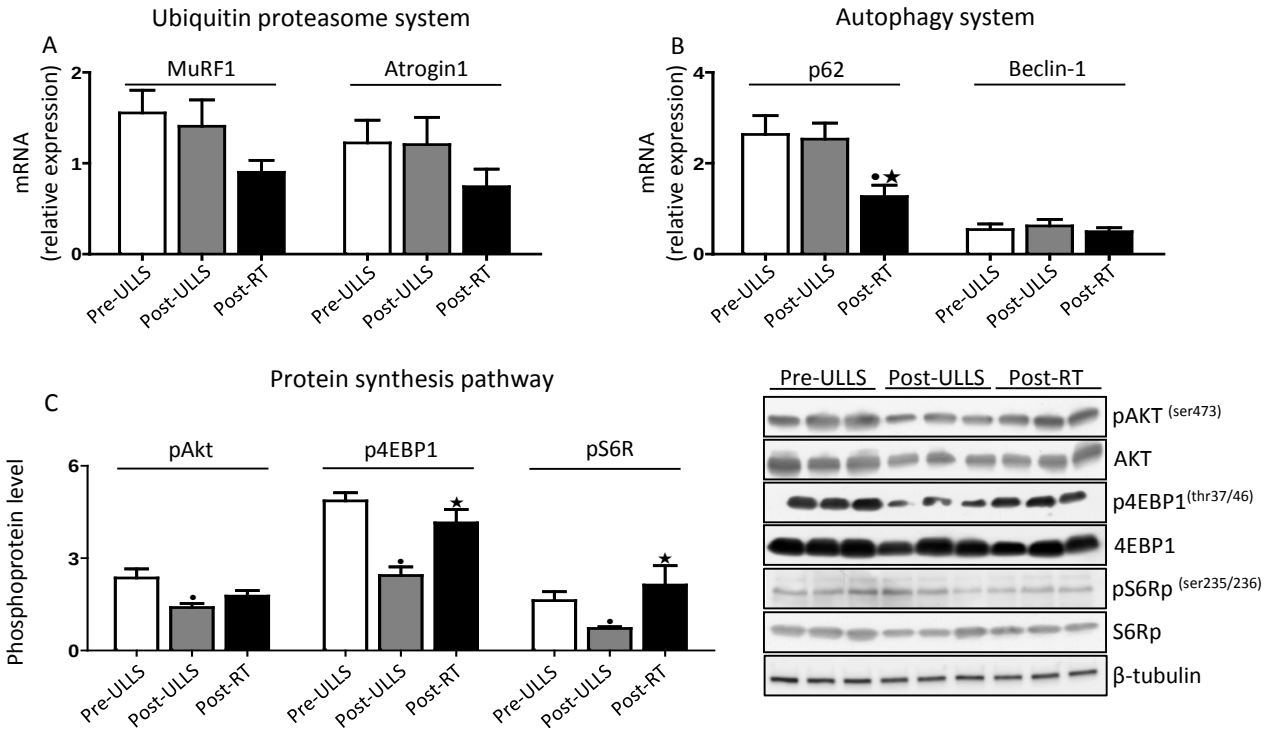
Ubiquitin proteasome system
A
MuRF1
Atrogin1
mRNA (relative expression)
Pre-ULLS
Post-ULLS
Post-RT
Autophagy system
B
p62
Beclin-1
Protein synthesis pathway
C
pAkt
p4EBP1
pS6R
Phosphoprotein level
Pre-ULLS
Post-ULLS
Post-RT
pAKT (ser473)
AKT
p4EBP1(thr37/46)
4EBP1
pS6Rp (ser235/236)
S6Rp
β-tubulin

Fig. 9

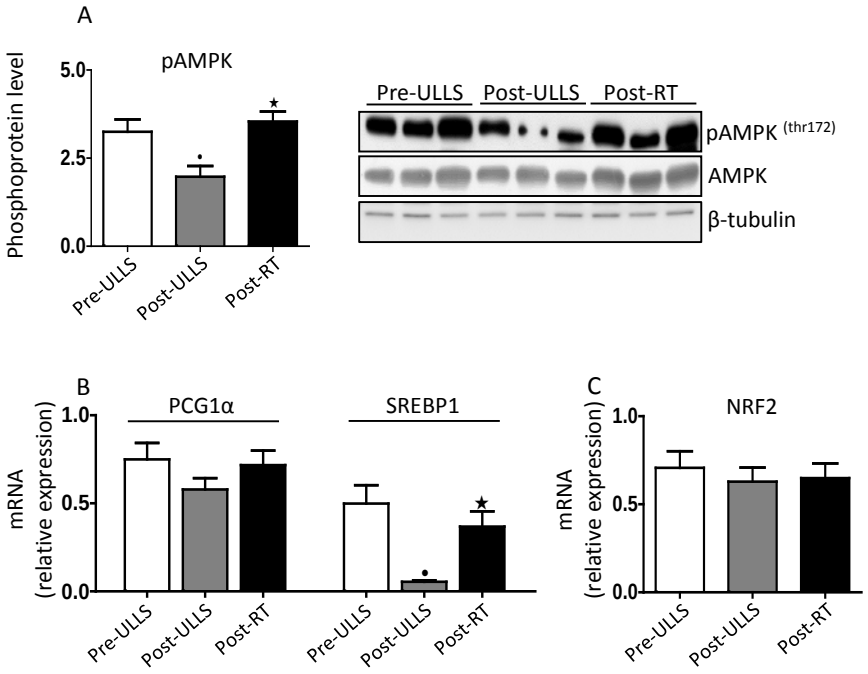
A
pAMPK
Phosphoprotein level
5.0
2.5
0.0
Pre-ULLS
Post-ULLS
Post-RT
Pre-ULLS
Post-ULLS
Post-RT
pAMPK (thr172)
AMPK
β-tubulin
B
PCG1α
SREBP1
mRNA
(relative expression)
1.0
0.5
0.0
Pre-ULLS
Post-ULLS
Post-RT
Pre-ULLS
Post-ULLS
Post-RT
C
NRF2
mRNA
(relative expression)
1.0
0.5
0.0
Pre-ULLS
Post-ULLS
Post-RT

Fig. 10

	Proteins	Abbreviations	Acces number	Estimated pI in 2D gels	Estimated MW in 2D gel	SCORE
	Myofibrillar proteins					
1	Troponin I fast	TnIf	P48788	8.9	21	124
2	Troponin I fast	TnIf	P48788	8.9	21	88
3	Troponin I fast	TnIf	P48788	8.9	21	246
4	Troponin I fast	TnIf	P48788	8.9	21	263
5	Troponin I fast	TnIf	P48788	9.36	21	297
6	Troponin T fast	TnTf	**P45378**	6.07	30	429
7	Tropomyosin alpha chain	Tpm1	P09493	4.7	33	203
8	Myosin light chain 2, fast	MLC-2f	Q96A32	4.79	18	98
9	Myosin light chain 3, fast	MLC-3f	P05977	4.6	16	293
10	Actin	Act	P68133	5.10	44	350
11	Myozenin-1	Myoz-1	Q9NP98	7.1	32	179
	Energy production system					
	Glycolytic metabolism					
12	Triose phosphate isomerase	TPI	P60174	6.7	26	89
13	Triose phosphate isomerase	TPI	P60174	6.7	26	788
14	Triose phosphate isomerase	TPI	P60174	6.7	26	150
15	Triose phosphate isomerase	TPI	P60174	6.7	26	547
16	Triose phosphate isomerase	TPI	gelfi	6.7	26	152
17	β enolase	ENO	P13929	6.8	53	531
18	β enolase	ENO	P13929	6.8	53	148
19	β enolase	ENO	P13929	7.59	47	786
20	β enolase	ENO	P13929	7.6	47	656
21	Lactate dehydrogenase A	LDHA	P00338	8.4	37	97

22	Lactate dehydrogenase A	LDHA	P00338	7.7	32	198
23	Lactate dehydrogenase A	LDHA	P00338	7.6	31	227
24	Aldolase A	ALDOA	P04075	8.5	40	528
	Oxidative metabolism					
25	NADH dehydrogenase Fe-S protein 3	NDUFS3	O75489	7.0	30	506
26	ATP synthase β chian	ATPβ	P06576	4.9	48	117
	Others metabolism					
27	Creatine kinase M-type	CK	Gelfi 2003			
28	Creatine kinase M-type	CK	Gelfi 2006			
29	Adenilate kinase 1	ADK1	P00568	8.7	22	282
30	Adenilate kinase	ADK	P00568	8.7	22	530
	Redox imbalance					
31	Superoxide dismutase 1	SOD1	P00441	5.8	19	320
32	Glutathione -*S*-transferase P	GSTP1	P09211	5.4	23	500
33	Glutathione-S-transferase Mu 2 isoform 1	GSTM1	P28161	6.0	26	902
34	Carbonic anhydrase 3	CA3	P07451	6.9	30	198
35	Peroxiredoxin 3	PRDX3	P30048	6.2	23	247
36	αβ-crystallin	αβ-cry	P02511	6.7	20	408
37	αβ-crystallin	αβ-cry	P02511	6.7	20	213
38	αβ-crystallin	αβ-cry	P02511	6.7	20	588
39	Heat shock protein beta1	HSPb1	P04792	6.3	23	
40	Heat shock protein beta1	HSPb1	P04792	6.3	23	444
	Others proteins					
41	Proteasome subunit alpha isoform 2	Prot-α-sub	P25787	6.2	30	142
42	Four and a half LIM domains protein 1	FHL1	Q13642	9.2	36	56
43	Parkinson disease	DJ-1	Q99497	6.3	20	56

	protein7					
44	Elongation factor Tu, mitochondrial	EF-tu	P49411	Gelfi 2003		