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Human skeletal muscle type 1 fibre distribution and response of stress sensing proteins along the titin molecule after submaximal exhaustive exercise

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ABSTRACT

Early responses of stress sensing proteins, muscle LIM protein (MLP), ankyrin repeat proteins (Ankrd1/CARP and Ankrd2/Arpp) and muscle-specific RING finger proteins (MuRF1 and MuRF2), along the titin molecule were investigated in the present experiment after submaximal exhaustive exercise. Ten healthy men performed continuous drop jumping unilaterally on a sledge apparatus with a submaximal height until complete exhaustion. Five stress sensing proteins were analysed by mRNA measurements from biopsies obtained immediately and 3 hours after the exercise from exercised vastus lateralis muscle while control biopsies were obtained from non-exercised legs before the exercise. Decreased maximal jump height and increased serum creatine kinase activities as indirect markers for muscle damage and HSP27 immunostainings on muscle biopsies as a direct marker for muscle damage indicated that the current exercised protocol caused muscle damage. mRNA levels for four (MLP, Ankrd1/CARP, MuRF1 and MuRF2) out of the five studied stress sensors significantly ($p<0.05$) increased 3 hours after fatiguing exercise. The magnitude of MLP and Ankrd2 responses was related to the proportion of type 1 myofibres. Our data showed that the submaximal exhaustive exercise with subject's own physical fitness level activates titin-based stretch-sensing proteins. These results suggest that both degenerative and regenerative pathways are activated in very early phase after the exercise or probably already during the exercise. Activation of these proteins represents an initial step forward adaptive remodelling of the exercised muscle and may also be involved in the initiation of myofibre repair.

Keywords: Human skeletal muscle, exercise induced muscle damage, titin, stress sensing proteins

INTRODUCTION

The early responses of stress sensing proteins along the titin molecule, were studied in the present experiment after fatiguing exercise on subjects own physical fitness level. A single titin molecule extends over half of a sarcomere, with its N-terminus located in the Z-disc, and the C-terminus located in the M-line (Tskhovrebova and Trinick 2003). To date, three mechanosensory "hotspots" along the titin molecule have been identified. These stress sensors are located at the Z-disc, N2A or N2B region of the I-band and the M-line (Lange et al. 2006, Kojic et al. 2011). At the Z-disc, in the N-terminus of the titin, muscle LIM protein (MLP, also known as cysteine rich protein 3 (CSRP3)) has been suggested to be a key putative hypertrophic mechanosensors (Knöll et al. 2002). Whereas in the I-band the muscle ankyrin repeat protein (MARP) family members, Ankrd1/CARP and Ankrd2/Arpp, are thought to function as messengers in stress response pathways (Miller et al. 2003). Similarly, muscle-specific RING finger proteins 1 and 2 (MuRF1 and MuRF2) in the C-terminus of titin at the M-line have been suggested to sense mechanical forces and regulate atrophic response (Centner et al. 2001). All of these protein complexes bind titin in the sarcomere, except in response to mechanical stimuli when they appear to transfer to the nuclei (Lange et al. 2006).

While the effects of physical activity on these titin-based stretch-sensing complexes are not well studied, there are a few publications on both rodent and human skeletal muscles. mRNA and protein levels of MLP respond quickly to mechanical loading (Schneider et al. 1999) and unloading (Roberts et al. 2012). Experiments on MLP knockout mice showed that MLP has an important role in upregulating MyoD activity in response to eccentric contraction-induced injury and in the maintenance of normal muscle function by affecting fiber size and resting sarcomere length (Barash et al. 2005). Ankrd1/CARP expression is low in adult skeletal muscle (Miller et al. 2003), however its expression has been shown to be elevated by exercise (Hentzen et a. 2006, Lehti et al. 2009), denervation (Tsukamoto et al. 2002) and muscle diseases such as muscular dystrophy (Laure et al. 2009). Ankrd2/Arpp is mainly expressed in slow type I fibers in adult skeletal muscles (Tsukamoto et al. 2002) and exercise has also been shown to increase Ankrd2/Arpp expression (Hentzen et a. 2006, Lehti et al. 2009). However, in type 1 diabetic mouse model physical activity decreased the elevation of Ankrd2/Arpp levels (Lehti et al. 2007). MuRF1 is elevated in a variety of muscle atrophy inducing conditions (Mayans and Labeit 2012). Knockout studies have shown that both MuRF1 and MuRF2 knockout mice have normal fertility and life span. Interestingly, MuRF1/MuRF2 double knockout mice develop an extreme and lifelong muscle hypertrophy (Witt et al. 2008).

The present exercise model, an exhausting stretch-shortening cycle (SSC) exercise on a sledge apparatus by continuous drop jumping was chosen to generate high mechanical loading on thigh muscles and to induce structural changes on muscle fibres. In jumping exercise, both eccentric and concentric muscle contractions are present as the muscles undergo repeated stretch-shortening cycles. In order to induce and to study skeletal muscle damage in humans, maximal eccentric exercise with the knee-extensors or elbow flexors on an isokinetic dynamometer has been frequently used. In these single joint eccentric exercise protocols, the muscle force-generating capacity typically decreases immediately post-exercise

on average a 50 % followed by gradual recovery over the next days or weeks (Paulsen et al. 2013, Clarkson and Hubal 2002). In these experiments, each subject performs the same number of muscle actions, which appears to result in clear individual differences both in force-generating capacity immediately post-exercise and in the length of the force recovery period (Paulsen et al. 2013, Sayers and Clarkson 2001). It is not known why the same amount of eccentric exercise induces skeletal muscle fibre damage, loss in force-generating capacity and prolonged force recovery period for some individuals whereas only short term decrease in muscle force-generating capacity was observed in other subjects (Paulsen et al. 2013). In the present study, each subject performed continuous drop jumping on a sledge apparatus with submaximal height until complete exhaustion. This was to ensure that each subject executed the exercise relative to their own maximal physical fitness level.

The current physical activity recommendations are based on duration of physical activity rather than the intensity of the exercise or individual physical fitness level. The goal of the present study was to investigate whether submaximal exhaustive exercise initiate the early responses of stress sensing proteins along the titin molecule located in three different stress sensing areas of the sarcomere. Fistly, muscle damage was monitored at three levels, functional, systemic and tissue level; decreased maximal jump height, increased serum creatine kinase activity and appearance of heat shock protein 27 (HSP27) immunostaining on muscle biopsies from the vastus lateralis muscle, respectively. HSP27 participates to protein stabilization during skeletal muscle fibre damage by saving proteins from unfolding. In addition to that α-actinin-3 deficiency was tested with immunostainings. α-actinin-3 is expressed predominantly in fast type 2 muscle fibres and circa 16% of the global human population is completely α-actinin-3 deficient (North et al 1999). Finally, stress sensing responses were analysed by mRNA measurements for the titin-based stretch-sensing proteins from Z-disk (MLP), I-band (Ankrd1/CARP and Ankrd2/Arpp) and M-line (MuRF1 and MuRF2) from biopsies immediately and 3 hours after the exercise.

METHODS

Ethical approval

The present study was approved by the ethics committee of the Healthcare District of Central Finland (ID 34/2007) and conformed to the standards set by the Declaration of Helsinki.

Subjects

Ten healthy, physically active men (26±6 years, 77.8±11.2 kg, mean ± SD) gave their written, informed consent to participate in the study. The subjects followed a normal diet and did not take any form of medication. None of the subjects were involved in systematic training during the past year. The subjects fasted overnight before the exercise. Caloric restriction was performed during measurements by taking only two chocolate bars (180 kcal/100g) before and after the fatiguing exercise protocol.

Baseline measurements of maximal isometric voluntary muscle contraction and maximal height of drop jump from 40 cm

Subjects warmed-up for 5-10 minutes on a bicycle ergometer. Thereafter the subjects were seated to the sledge apparatus. The sledge was inclined 23° from the horizontal level. The subjects' knee angle was set to 107°. The maximal isometric voluntary muscle contraction of the leg extensors was performed unilaterally by pressing maximally the force plate. Three attempts were recorded with both legs. Next, the subjects performed 10 maximal unilateral drop jumps on both legs from the height of 40 cm at a frequency of one every fifth second. Subjects were instructed to jump with their maximal effort for obtaining the highest bound.

Fatiguing exercise protocol

Fatigue was induced by performing continuous drop jumping on a sledge apparatus with a submaximal height, which was 50% of the height of the individual highest bound tested before the exercise, until complete exhaustion. The exercise was performed on the right leg, while the left leg served as a control. The sledge was inclined 23° from the horizontal level.

Postexercise measurements of maximal isometric voluntary muscle contraction and maximal height of drop jump from 40 cm

Maximal isometric voluntary muscle contraction (data is not shown here) and maximal height of drop jump from 40 cm were tested immediately after, 3 hours and 1 day after the exercise. Testing was done the same way as described above.

Whole blood lactate and serum creatine kinase analyses

The blood samples were collected 3 hours before the fatiguing exercise and immediately, 3 hours and 1 day after the exercise. The whole blood lactate samples from finger tips were analysed with Biosen C_line (EKF Diagnostic GmbH, Barleben, Germany) and serum (venous blood) creatine kinase activity according to manufacturer's instruction (Konelab™ System CK, Thermo Fisher Scientific Inc., Waltham, MA USA) with Konelab 20XTi (Thermo Fisher Scientific Inc.).

Muscle biopsies

Needle muscle biopsies were obtained under local anesthesia (Lidocain 20 mg/ml c.adrenalin, Orion Pharma, Espoo, Finland) 3 hours before the exercise from the middle region of the non-exercised vastus lateralis muscle, and immediately and 3 hours after the exercise from the exercised vastus lateralis muscle. The histology samples were mounted in the Tissue-Tek® O.C.T. Compound (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and frozen in isopentane cooled in liquid nitrogen. The samples for RNA analyses were frozen in liquid nitrogen. All samples were stored at -80ºC until further analysis

Immunohistochemical stainings of skeletal muscle sections

Ten μm thick serial sections were cut on a cryostat microtome (Leica CM3000, Leica Biosystems, Nussloch GmbH, Germany) at -24°C. Muscle cross-sections were immunohistochemically stained with Heat Shock Protein (HSP) 27 antibody (dilution 1:250, CPTC-HSPB1-1, mouse monoclonal, Developmental Studies Hybridoma Bank, University of Iowa, Department of Biology, Iowa, USA) for localization of structural damage in the skeletal muscle fibres, with dystrophin antibody (1:500, ab15277, rabbit polyclonal, Abcam, Cambridge, UK or 1:200, NCL-DYS2, mouse monoclonal, Novocastra, Leica Biosystems, Nussloch, Germany) for visualization of borders of muscle fibres, with slow myosin heavy chain antibody for counting the type 1 fibre proportion (1:200, BA-F8, mouse monoclonal, Developmental Studies Hybridoma Bank) and with α-actinin-3 (1:500, ACTN3, ab68204, rabbit monoclonal, Abcam) for identifying subjects, who are α-actinin-3 deficient. All dilutions were made in PBS. Sections were washed for 5 minutes in PBS, fix in 4% paraformaldehyde or Histofix (Histolab Products AB, Göteborg, Sweden) permeabilized in 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, USA) for 10 minutes, blocked with 5% goat serum (Gibco, Thermo Fisher Scientific Inc.) for 30 minutes at room temperature and incubated overnight with primary antibody dilution in 1% goat serum at 4 C°. After washing the slides for 10 minutes in PBS the sections were incubated for 60 minutes with Alexa Fluor® 488 or 555 goat anti—mouse IgG and goat anti-rabbit IgG secondary antibody (Molecular Probes, Thermo Fisher Scientific Inc.) diluted 1:200 in 1% BPS at room temperature. After washing the fluorchrome-stained sections for 5 minutes in PBS, nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, dilactate, Invitrogen, Thermo Fisher Scientific Inc.) and the sections were mounted on Prolong® Diamond Antifade Mountant mounting medium (Molecular Probes, Thermo Fisher Scientific Inc.). Muscle sections stained without primary antibody were used as negative controls.

Evaluation of HSP27 stained muscle sections

Under the normal conditions HSP27 is localized to the Z-disks of skeletal muscle fibres (Kötter et al. 2014, Figure 1A, B), whereas after eccentric exercise HSP27 is accumulated to variable-sized clusters (Paulsen et al. 2009, Figure 1C-F). The appearance of HSP27 stained clusters inside skeletal muscle fibres was evaluated by scoring the sections on three levels; 1, 2 and 3. Scoring criteria for level 1 was that at least one clearly HSP27 stained cluster (such as in Figure 1C) was observed inside skeletal muscle fibre. Criteria for level 2 were that approximately half of the fibres had at least one clearly HSP27 stained cluster or that approximately half of the total fibre area contained HSP27 stained clusters. Criteria for level 3 was that almost all fibres contained at least one clearly HSP27 stained cluster. Each subject had one biopsy before the exercise and two biopsies, immediately and 3 hours, after the exercise.

mRNA analysis

Muscle total RNA was isolated with TRI Reagent ® Solution (Applied Biosystems, Foster City, CA, USA) using FastPrep homogenizer and FastPrep Green tubes (Qbiogen, Carlsbad, CA, USA). The isolation steps were performed according to the manufacturer's instructions. Total RNA concentration and purity were determined by measuring absorbance at 260 nm and 280nm with spectrophotometer ND-1000 (Nano Drop, Thermo Fisher Scientific Inc.).

Total RNA was reverse transcribed using a high-capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. Amount of mRNA of studied proteins was measured by ABI Prism 7300 Real Time PCR system (Applied Biosystems). TaqMan Universal PCR Master Mix and TaqMan Gene Expression assays (Applied Biosystems) Ankrd1/CARP (Hs00173317_m1), Ankrd2/Arpp (Hs00220469_m1), MuRF2 (Hs00979327_m1) and GAPDH (Hs99999905_m1). Primers for MLP (For: For: 5'-GTC CGA GAA GTG CCC TCG ATG-3', 5'-GTA AGG CCT CCA AAC CCA AT-3') and MuRF1 (For: 5'-GCTCAGAGAGCAGGGACTAG-3', Rev:5'-AAAGCACCAAATTGGCATAC-3') were designed in house and the amount of mRNA was analysed with iTaq Universal SYBR Green Supermix. Expression of mRNA of studied proteins was normalized to GAPDH expression.

Statistics

A non-parametric equivalent of variance analysis for related samples, Friedman test, was used to search for statistically significant differences over time for the maximum jump height, creatine kinase and mRNA levels for MLP, Ankrd1/CARP, Ankrd2/Arpp, MuRF1 and MuRF2. The statistical significances of the time points compared to baseline was determined by non-parametric Dunn's multiple comparisons test for two related samples, when Friedman's test showed $p < 0.05$. GraphPad Prism 6.03.

A simple linear regression analysis was run to determinate correlations and statistical significances for selected variables such as type 1 fibre proportion and mRNA levels for MLP

or Ankrd2/Arpp mRNA levels related to baseline, type 2 fibre proportion, creatine kinase activity or jump height and MuRF1 mRNA level related to baseline. SPSS version 24.

RESULTS

Drop jumping and individual characteristics of the subjects

In the current study, each subject performed continuous drop jumping on a sledge apparatus with submaximal height until complete exhaustion. Duration of the jumping varied from 1 minute 43 seconds (61 jumps) to 30 minutes 5 seconds (1248 jumps). Individual characteristics of the subjects including maximal jump height, maximal voluntary force before the exercise and type I fibre percentage as well as the performance results such as the duration of the jumping, the number of jumps and the whole blood lactate immediately after the exercise are presented in Table 1. Furthermore, one of ten subjects showed no α-actinin-3 staining (Supplemental Figure 1, Table 1). The α-actinin-3 deficient subject was one of four subjects showing slow recovery 1 day after the exercise (the maximal jump height was 67 % of the baseline) and had high serum creatine kinase (656 U/l) before the exercise, low lactate (3.3 mmol/l) immediately after the exercise and the highest individual increase in mRNA level for MLP.

Maximal jump height

Friedman test showed statistically significant differences over time for the maximum jump height ($p<0.001$). Further statistical evaluation by non-parametric Dunn's multiple comparisons test showed statistically significant differences immediately ($p<0.001$) and 3 hours ($p<0.01$) after the drop jumping compared to the maximum jump height before the exercise. There was no statistically significant difference in 1 day after the exercise compared to baseline results. However, slower recovery (maximal jump height was 61-85% of the baseline) was observed in four out of the nine subjects. Individual results are presented in Supplemental Table 1.

Creatine kinase activity

Friedman test showed statistically significant differences over time for creatine kinase values ($p<0.001$). Further statistical evaluation by non-parametric Dunn's multiple comparisons test showed statistically significant differences 3 hours ($p<0.001$) and 1 day ($p<0.001$) after the drop jumping compared to creatine kinase values before the exercise. There was no statistically significant difference in immediately after the exercise compared to baseline results. Individual results are presented in Supplemental Table 2.

HSP27 immunostainings

In biopsies obtained before the exercise, no HSP27 stained clusters were observed. HSP27 was localized to the Z-disc and/or I-band of skeletal muscle fibres (Figure 1A, B) as it is reported for example by Kötter et al. 2014. Whereas after the exercise intensively stained and variable-sized clusters of HSP27 (Figure 1C-F) were observed at least one of the two post-exercise biopsies in every subject. Three out of ten subjects showed level 3 staining (almost all fibres contained at least one clearly HSP27 stained cluster), whereas six out of ten subjects showed level 1 staining (at least one clearly HSP27 stained cluster was observed) and in

addition one out of ten subjects showed level 2 staining. Individual HSP27 scoring results are presented in Supplemental Table 3.

mRNA levels for the titin-based stretch-sensing proteins MLP, Ankrd1/CARP, Ankrd2/Arpp, MuRF1 and MuRF2

Friedman test showed statistically significant differences over time for MLP ($p<0.05$), Ankrd1/CARP ($p<0.001$), Murf1 ($p<0.01$) and Murf2 ($p<0.001$) (Figure 2). Further statistical evaluation by non-parametric Dunn's multiple comparisons test showed statistically significant increase in 3 hours after the drop jumping compared to before the exercise for MLP ($p<0.05$), Ankrd1/CARP ($p<0.01$), Murf1 ($p<0.01$) and Murf2 ($p<0.001$). There were no statistically significant differences in immediately after the exercise compared to baseline results. Friedman test did not show statistically significant difference over time for Ankrd2. Summary of statistical analysis is presented in Table 2.

A simple linear regression analysis (Table 3) showed strong positive correlation and statistically significant relationship between MLP mRNA levels related to baseline, immediately after the exercise and type 1 fibre proportion ($r=0.79$, $p=0.011$). The relationship between Ankrd2 mRNA level related to the baseline, 3 h after the exercise and type 1 fibre proportion had also strong positive correlation and was statistically significant ($r=0.753$, $p=0.031$), whereas mRNA level for Ankrd2 related to baseline, immediately after exercise showed moderate correlation ($r=0.548$), but was not statistically significant. MuRF1 mRNA level related to baseline, immediately after the exercise and creatine kinase activity 1 day after the exercise showed strong positive correlation and was statistically significant ($r=0.73$, $p=0.039$). Whereas with jump height 3h after the exercise, MuRF1 mRNA levels showed strong negative correlation ($r= -0.75$, $p=0.019$).

DISCUSSION

Skeletal muscle damage

In the present study, skeletal muscle damage was evaluated from changes in maximal jump height, serum creatine kinase activity and HSP27 immunostaining on muscle biopsies after the exercise. All of these three makers showed rapid and considerable responses to exercise suggesting that fatiguing exercise induced structural damage in skeletal muscle. The current exercise protocol, continuous drop jumping on a sledge apparatus with submaximal height until complete exhaustion, ensured that each subject executed the exercise on their own maximal physical fitness level. Maximal jump height decreased statistically significantly immediately and 3 hours after the drop jumping compared to the maximal jump high before the exercise. Whereas, no statistically significant differences were observed 1 day after the exercise compared to baseline results. However, four out of the nine subjects showed slower recovery (maximal jump height was 61-85% of the baseline, Supplemental Table 1). Similar decrease and slower recover for few subjects in the muscle force-generating capacity have been observed in the single joint eccentric exercise protocols (Paulsen et al. 2013, Clarkson and Hubal 2002).

Serum creatine kinase activity has traditionally been used as a marker for skeletal muscle damage (see for review Koch et al. 2014). The present exercise protocol increased serum creatine kinase activity statistically significantly 3 hours and 1 day after the exercise. Serum creatine kinase activity typically peaks at 2-4 days after the exercise (Koch et al. 2014). The latest time point analysed in the present study was 1 day after the exercise (ranged from 600 to 2000 U/l). Therefor the peak serum creatine kinase values are probably not observed yet at 1 day after the exercise. However, the serum creatine kinase activity values as high as 2000 U/l suggested that fatiguing exercise may have caused some muscle damage.

Fine lines of HSP27 immunostainings was observed in longitudinal sections of unexercised skeletal muscle fibres (Figure 1A) indicating that HSP27 was localized to the Z-disks and/or I-band (Paulsen et al 2009). After the exercise HSP27 immunostainings showed intensively stained and variable-sized clusters in both cross-sectional and longitudinal sections of skeletal muscle fibres HSP27 (Figure 1C-F), as it has been reported previously (for example by Paulsen et al. 2009). These stained clusters were probably formed due to translocation and accumulation of HSP27 on cytoskeletal/myofibrillar structures (Paulsen et al. 2009). In general, HSPs are considered to be the cellular protein quality control machinery. They can stabilize proteins during cellular damage, contribute to protein folding during increased protein synthesis and protect proteins from aggregation (Mymrikov et al. 2011). HSP27 can interact with actin and with many actin-binding proteins such as tropomyosin and troponin T (Mymrikov et al. 2011). Interestingly, it has been recently shown that HSP27 can be associated with immunoglobulin-like (Ig) domain of the titin springs (Kötter et al. 2014) Kötter et al. (2014) showed that aggregation of unfolded titin Ig domains stiffens myocyte and that HSP27 translocation to these domains may prevent this aggregation. In summary, the changes in two indirect markers, maximal jump height and serum creatine kinase activity together with HSP27 immunostainings on muscle biopsies as a direct marker for muscle

damage indicated that the fatiguing exercise on subject's own maximal physical fitness level caused some muscle damage.

Stress sensors along the titin molecule

Titin is a potential candidate to sense alterations in mechanical loading due to its central and longitudinal position within the sarcomere and due to its large size. There are three known protein interaction sites, which are thought to sense mechanical loading along the titin molecule (Krüger and Kötter 2016). In the present experiment, mRNA levels for four out of the five studied stress sensors at the Z-disc, the elastic I-band and the M-band showed statistically significance increase 3 hours after fatiguing exercise (Table 2).

MLP at the Z-disc

The Z-disc anchors the ends of actin filaments and transmits forces from one sarcomere to the next. In addition to the structural role of the Z-disc, it acts as a stretch sensor and is a nodal point for mechano-signaling networks. The NH_2-terminal end of titin is anchored in the Z-disc via nebulin, α-actinin 2 and telethonin. A complex formed by MLP, titin and telethonin has been suggested to act as a mechanical stress sensor (Lange et al. 2006). In the present study, mRNA level for MLP increased statistically significantly already 3 hours after the drop jumping compared to before the exercise. No statistically significant difference was observed for MLP mRNA level immediately after the exercise compared to baseline results. It has previously been shown on human skeletal muscle that acute exercise increases MLP mRNA levels 6 h after eccentric stepping exercise compared to contralateral leg, which performed concentric stepping exercise (Kostek et al. 2007) and 2 days after fatiguing jumping exercise (Lehti et al. 2009). Both of these exercise protocols, although been also fatiguing exercise, contained maximal muscle contractions such as stepping exercise with extra weights (Kostek et al. 2007) and 100 maximal unilateral drop jumps (Lehti et al. 2009), whereas our exercise protocol contained only submaximal exercise and was more endurance type of exercise.

According to animal experiments MLP is present at high levels in adult rat slow-twitch muscles, whereas it is not detectable in fast-twitch muscles (Schneider et al. 1999). However, MLP expression can be induced to fast-twitch muscles by increased neuromuscular activity using chronic low-frequency stimulation (Schneider et al. 1999). To our knowledge the results of the present study showed for the first time that increased MLP mRNA levels immediately after the exercise were dependent on proportion of type I fibres in humans (Table 3). Interestingly, the highest individual increase in mRNA level for MLP was observed in the α-actinin-3 deficiency subject. Both α-actinin-3 and MLP are located in the Z-disc of a sarcomere. According to knock-out mouse studies, Z-disc proteins are more highly expressed but normally localized with a-actinin-3 deficiency (Seto et al. 2011). Our results showed rapid and slow muscle fibre related increase in MLP expression after the exercise, suggesting that fatiguing exercise on subjects own physical fitness initiates the early responses of stress sensing protein MLP.

Ankrd1/CARP and Ankrd2/Arpp at the N2A region of the I-band

In the I-band, titin is mostly composed of Ig-domains including the PEVK and the N2A domains. The I-band part of titin represents the main elastic segment of titin, which can be sequentially extended during sarcomere stretch. Titin's skeletal muscle specific N2A-domain has been shown to interact with Ankrd1/CARP and Ankrd2/Arpp (Miller et al. 2003). In the present study Ankrd1/CARP showed statistically significant increase in 3 hours after the drop jumping compared to before the exercise (Table 2). A pronounced increase of Ankrd1/CARP mRNA level has been observed previously in human skeletal muscle as early as 30 minutes postexercise (Lehti et al. 2009). No statistically significant differences were observed for Ankrd1/CARP and Ankrd2/Arpp mRNA levels immediately and for Ankrd2/Arpp 3h after the exercise compared to baseline results.

Ankrd1/CARP is expressed primarily in cardiac muscle. In normal skeletal muscle, both mRNA and protein levels for Ankrd1/CARP are nearly undetectable. Similarly in the present study, the mRNA level for Ankrd1/CARP of unexercised skeletal muscle was barely detectable, whereas in exercised muscle the increase was clearly pronounced. According to animal models, Ankrd1/CARP is considered as a hypertrophic gene (see for example Anihara et al. 2000 for the cardiac hypertrophy model and Carson et al. 2002 for the work overload model in skeletal muscle). Unlike Ankrd1/CARP, Ankrd2/Arpp is expressed in normal skeletal muscle preferentially in slow type I muscle fibres (Pallavicini et al 2001, Tsukamoto et al. 2002). Our study showed strong positive correlation between increased Ankrd2/Arpp mRNA levels related to baseline level and higher type 1 fibre proportion 3 hours after the exercise, whereas immediately after the exercise the correlation was moderate. This is in line with finding from animal studies that stretch increased Ankrd2 level are related to slow type muscles and/or muscle undergoing transformation towards slower fibre types (Mckoy et al. 2005, Tsukamoto et al. 2002). Jasnic-Savovic et al. (2016) has recently reported the expression of two ANKRD2 isoforms. The function of these isoforms is unknown at the moment. Our PCR primers are expected to recognize both of these isoforms. Like MLP, ANKRD2 showed slow muscle fibre related increase after the exercise. Moreover the hypertrophic gene ANKRD1 increased 3 hours the exercise. These findings strengthen the observation that the current exercise can initiate the early responses of stress sensing proteins.

Murf1 and Murf2 at the M-line

In the M-line titin contains a kinase domain near the C-terminus, which forms protein complexes with MuRF1 (Centner et al. 2001) and with nbr, p62 and MuRF2 (Lange et al 2005). MuRF1 is a mediator of muscle waste and is upregulated by atrophic stimuli such as immobilization, denervation, nutritional deprivation, aging and disease (Bodine et al. 2001). MuRF1 has been characterized in more detail than MuRF2. Both MuRF1 and MuRF2 are involved in the transmission of mechanical forces to nuclear cell signaling pathways through their association with the sarcomere. According to the MuRF1- and MuRF2-deficient mouse

models, MuRF1 and MuRF2 can collaboratively control skeletal muscle mass and metabolism by regulating a large set of same proteins including proteins required for myofibrillar stretch sensing, translation, and transcription factors (Witt et al. 2008). In the present study both MuRF1 and MuRF2 showed statistically significant increase in 3 hours after the drop jumping compared to before the exercise (Table 2). No statistically significant differences were observed for MuRF1 and MuRF2 mRNA levels immediately after the exercise compared to baseline results.

In mouse skeletal muscle MuRF1 is predominantly expressed in fast type 2 fibres (Moriscot et al. 2010). Based on the results from MuRF1 and MuRF2 knock-out mice studies, it has been suggested that MuRF1 is required for remodelling of type 2 fibres under pathophysiological stress situation such as denervation and in addition to that both MuRF1 and MuRF2 are required for maintenance of type 2 fibres (Moriscot et al. 2010). No correlations were found between type 2 fibre proportions and MuRF1 mRNA levels related to baseline immediately or 3 hours post exercise in the present study (Table 3).

Furthermore, the present experiment showed that higher MuRF1 mRNA levels immediately after the exercise were followed by higher serum creatine kinase activity values 1 day after the exercise and by lower jump height 3 hours after the exercise. According to MuRF1 knockout mice studies, MuRF1 is shown to adjust creatine kinase activities in skeletal muscles by regulating its turnover in vivo (Koyama et al 2008) and TNF-α-induced reduction in skeletal muscle force development increased MuRF1 expression (Adams et al. 2008). Our results are in line with these animal studies and further supports the role of MuRF1 in remodelling of skeletal muscle tissue.

Summary/conclusion

In summary, our data propose that the submaximal exhaustive exercise relative to subject's maximal physical fitness level activated titin-based stretch-sensing proteins, MLP, Ankrd1/CARP, MuRF1 and MuRF2 and the magnitude of the response for MLP and Ankrd2 seemed to be related to the proportion of type 1 fibres. The current exercise protocol caused both functional (decreased maximal jump height) and structural (clusters of HSP27 immunostainings on muscle biopsies) changes in skeletal muscles. These results suggest that both degenerative and regenerative pathways are activated in very early phase after the exercise or probably already during the exercise. Activation of these proteins probably represents an initial step forward adaptive remodelling of the exercised muscle and may also be involved in the initiation of fibre myorepair. Changes in studied variables were surprisingly similar considering variation on number of jumps performed, underlining the importance of observing subject's own fitness level when designing exercise recommendations. Further studies are needed to investigate protein expression and localization of these titin-based stretch-sensing proteins.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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Table 1. Individual characteristics of the subjects and the performance results.

Subject	Age years	Weight kg	Height cm	Jump height cm	Max force N	Type I fibres %	Duration	Jumps n	B-Lactate[2] mmol/l
S01	36	55.0	162	70	1078	50	3 min 30 s	167	11.3
S02	34	68.3	172	45	1148	45	9 min 43 s	416	10.4
S03	18	70.6	178	57	4018	40	5 min 34 s	217	11.0
S04	32	88.2	184	53	1819	48	1 min 43 s	61	2.9
S05	24	85.7	185	70	3584	71	30 min 5 s	1248	8.6
S06	27	86.9	183	57	3211	57	3 min 5 s	152	9.0
S07	18	73.5	182	72	2379	62	8 min 43 s	324	12.3
S08	22	76.8	187	56	3640	69	22 min 48 s	1038	5.7
S09[1]	22	85.2	178	61	1907	66	7 min 45 s	307	3.3
S10	22	83.6	187	46	1670	61	10 min 0 s	411	8.0

[1]α-actinin-3 deficient subject, [2]whole blood lactate immediately after the exercise

Table 2. Summary of statistical analysis: mRNA levels of stretch-sensing proteins

Compound	Location	Friedman test	Ctrl vs 0h*	Ctrl vs 3h*
MLP	Z disc	p<0.05	ns	p<0.05
Ankrd1/CARP	I-band	p<0.001	ns	p<0.01
Ankrd2	I-band	ns	ns	ns
Murf1	M-line	p<0.01	ns	p<0.01
Murf2	M-line	p<0.001	ns	p<0.001

*non-parametric Dunn's multiple comparisons test, ns - no statistical significance

Table 3. A simple linear regression analysis of selected variables.

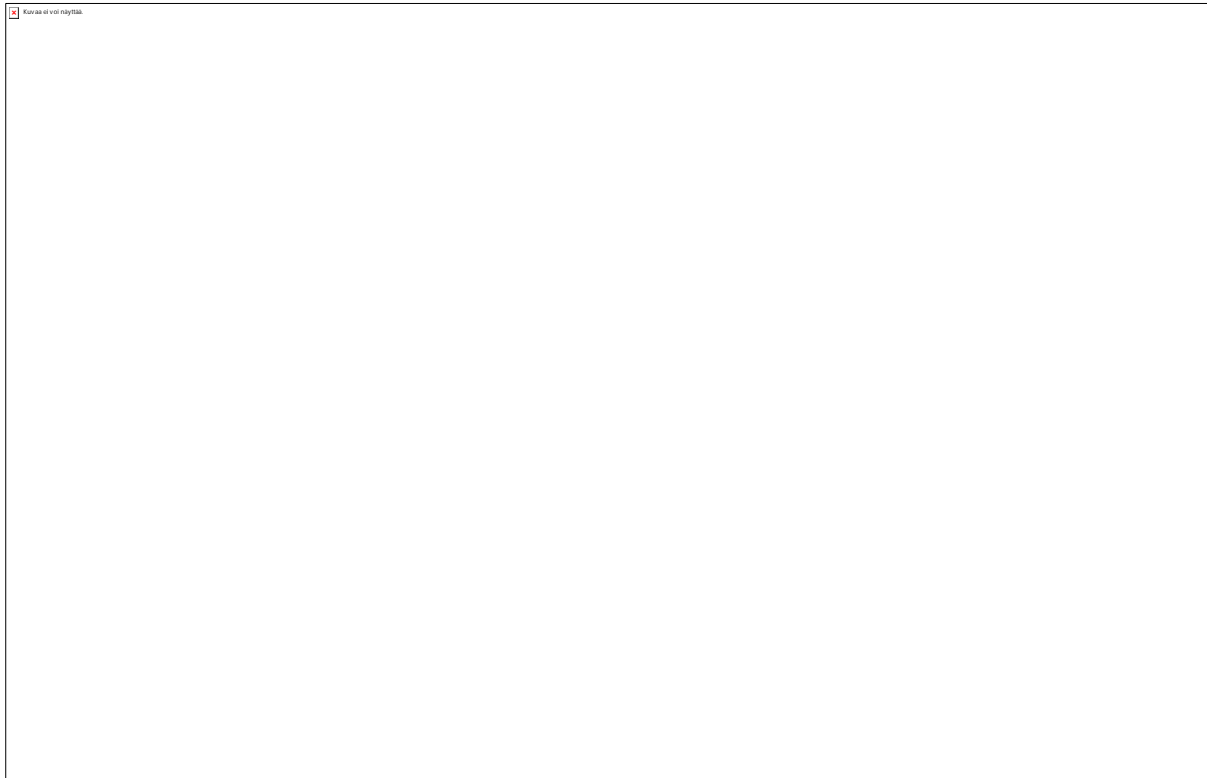| Dependent variable	Location	Independent variable	Correlation 0h	Correlation 3h
MLP	Z disc	type 1 fibre %	r= 0.79 (p=0.011)	r= 0.21 (ns)
Ankrd2	I-band	type 1 fibre %	r= 0.55 (ns)	r= 0.75 (p=0.031)
Murf1	M-line	type 2 fibre %	r= -0.14 (ns)	r= -0.32 (ns)
Murf1	M-line	creatine kinase 1d	r= 0.73 (p=0.039)	r= 0.64 (ns)
Murf1	M-line	jump height 3h	r= -0.75 (p= 0.019)	r= -0.21 (ns)

Figure 1. HSP27 (red) immunostaining as direct marker for muscle damage. In biopsies obtained before the exercise, no HSP27 stained clusters were observed (A and B). HSP27 was localized to the Z-disks of skeletal muscle fibers. After the exercise intensively stained and variable-sized clusters of HSP27 were observed at least one of the two post-exercise biopsies in every subject (C-F). The appearance of HSP27 stained clusters inside skeletal muscle fibres was evaluated by scoring the sections on three levels; 1, 2 and 3. Six out of ten subjects showed level 1 staining (at least one clearly HSP27 stained cluster was observed), whereas three out of ten subjects showed level 3 staining (almost all fibres contained at least one clearly HSP27 stained cluster) and one of the ten subjects had level 2 staining (around half of the fibres contained at least one clearly HSP27 stained cluster). Dystrophin (green) immunostaining was used to visualized the borders of muscle fibres and DAPI (blue) stained nuclei.

Figure 2. mRNA levels for stretch-sensing proteins Ankrd1/CARP (A), MLP (B), MuRF1 (C) and MuRF2 (D) of individual subjects. au – arbitrary units

Supplemental Table 1. Maximal jump height (cm)

| Subject	before	immediately	3 hours	1 day	recovery %*
S01	70	33	38	43	61
S02	45	21	39	41	92
S03	57	38	53	54	94
S04	53	38	38	50	95
S05	70	54	44	47	67
S06	57	51	53	58	102
S07	72	40			
S08	56	45	52	48	85
S09	61	40	56	41	67
S10	46	34	40	44	95

* recovery % is the percentage of the maximal jump high 1 day after the exercise compared to the baseline

Supplemental Table 2. Serum creatine kinase (U/l)

Subject	before	immediately	3 hours	1 day
S01	163	430	665	1280
S02	261	449	650	1198
S03	83	258	405	608
S04	289	388	539	1083
S05	126	414	1033	1976
S06	107	288	414	731
S07	139	371	673	1233
S08	125	269	370	729
S09	656	841	970	1196
S10	90	243	308	590

Supplemental Table 3. Individual HSP27 immunostaining scoring results. No HSP27 stained clusters in any of the before exercise biopsies (marked as 0). Scoring criteria for level 1: at least one clearly HSP27 stained cluster (such as in Figure 1C) inside skeletal muscle fibre. Scoring criteria for level 2: approximately half of the fibres had at least one clearly HSP27 stained cluster or approximately half of the total fibre area contained HSP27 stained clusters. Scoring criteria for level 3: almost all fibres contained at least one clearly HSP27 stained cluster.

Subject	Before	Immediately	3 h
S01	0	1	2
S02	0	1	1
S03	0	1	0
S04	0	3	2
S05	0	2	3
S06	0	1	1
S07	0	3	-
S08	0	1	1
S09	0	1	1
S10	0	1	1
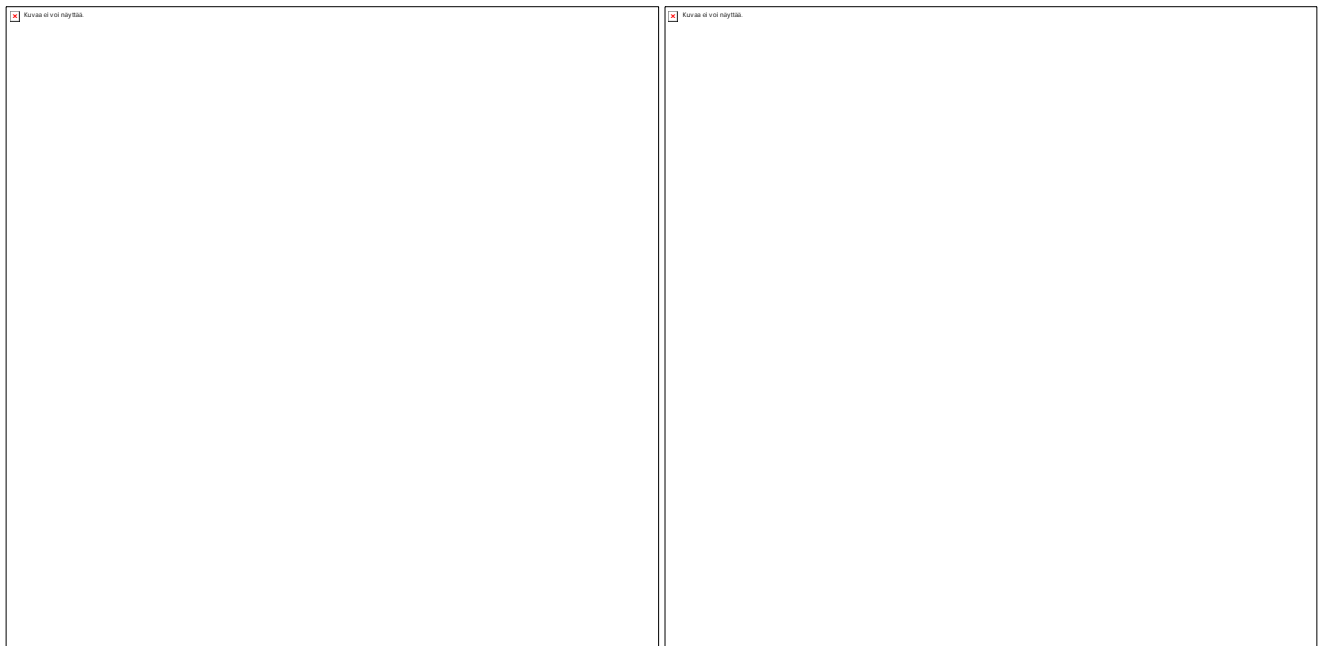
Supplemental Figure 1. Double staining with α-actinin 3 (red) and dystrophin (green).
A. Muscle biopsies of subject S09 showed no α-actinin 3 staining. **B.** The rest of the subjects showed similar staining pattern as can be seen from subject S03. α-actinin 3 is expressed predominantly in fast type 2 muscle fibres, whereas slow type 1 muscle fibres remained unstained .