Extracellular Matrix and Myofibrils During Unloading and Reloading of Skeletal Muscle

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Abstract

The aim of the study was to elucidate the effect of unloading and reloading on the collagen expression and synthesis rate of myofibrillar proteins in fast-twitch (FT) muscle in relation to changes in muscle strength and motor activity. Northern blot analysis was used for testing the specificity of cDNA probes and protein synthesis rate was measured according to incorporation of radioactive leucine into different protein fractions. Unloading depresses collagen type I and III (p < 0.001), type IV (p < 0.05) and reloading enhances collagen expression in fast-twitch skeletal muscle in comparison with unloading. Enhanced expression of matrix metalloproteinase-2 continued during the first week of reloading (p < 0.01) and tissue inhibitor of metalloproteinase-2 during reloading (p < 0.05). Changes in collagen expression in FT muscle are in good agreement with changes in myofibrillar protein synthesis during unloading and reloading. In conclusion alterations in extracellular matrix and myofibrillar apparatus in FT skeletal muscle are related to changes in muscle strength and motor activity, are significant in exercise training and determination of recovery periods in the training process as well as in athletes’ rehabilitation.

Introduction

It is well known that disuse (e.g. immobilization, denervation, muscle unloading, spaceflight, hind-limb suspension) results in skeletal muscle atrophy, a decrease in muscle fibres cross-sectional area, a reduction in myofibrillar protein content as a result of elevated proteolysis and increase in the percentage of fast myosin heavy chain (MyHC) isoforms [6,9,27,28,35]. Significant atrophy and contractile alterations occur in the slow-twitch (ST) muscles and only slight slow to fast transition in fast-twitch (FT) muscles [5,7,8,38]. Skeletal muscle contractile proteins and collagen change during unloading (reduced biomechanical loading) in a relatively short period [35]. It seems that changes in contractile structures of the skeletal muscle and collagen network lead to the depression of muscle function. Unloading decreases the total number of myonuclei and the mitotic activity of satellite cells [25,37] and activation of satellite cells is inhibited under the atrophic conditions. On the contrary, mechanical loading activates the regeneration, appearance of fibres with central nuclei, which were inhibited by unloading [23,30,31]. Loading also activates the extracellular matrix (ECM) which has multiple functions. Type I and III collagens are the most abundant fibrillar collagens in skeletal muscle. Nonfibrillar type IV collagen plays a role in the regenerative process of ECM, reorganization of the basement membrane components, including the matrix-associated signals and the membrane-associated receptors that underline muscle fibre – matrix interaction [20,29]. The role and dynamics of regulatory factors of ECM such as lysyl oxidase and matrix metalloproteinases during unloading and reloading in FT skeletal muscles are practically unknown.

Myopathic muscles, it has been shown that there are some similarities and differences between changes in contractile apparatus and ECM in fast and slow muscles [26]. Intracellular protein synthesis decreased only in FT muscles. In extracellular matrix the decrease of protein synthesis appears in both FT and ST muscles [26]. It has been shown in cerebral palsy patients that altered muscular control may influence ECM homeostasis, in particular, collagen [10]. Collagen turnover related gene expression profiles were found to be different in cerebral palsy patients in comparison with the control group.
European guidelines and ethical standards of the IJSM [13]. Tartu. All research and animal care was performed according to Committee of Laboratory Animals Science of the University of

maintained a normal colour, indicating that the blood regulatory processes to occur. The portion of exposed tail the tail remained uncovered, thereby allowing normal ther-

mals were anesthetized, so that the tail was easily manipulated. Globus [24] was used to suspend the hindlimbs of rats. The ani-

Hindlimb suspension procedure

A modification of the tail harness model of Morey-Holton and Globus [24] was used to suspend the hindlimbs of rats. The ani-

als were anesthetized, so that the tail was easily manipulated. The skin of the tail was wrapped in breathing tape so that half of the tail remained uncovered, thereby allowing normal thermoregulatory processes to occur. The portion of exposed tail maintained a normal colour, indicating that the blood flow was not compressed. A swivel harness was attached to the tail with a strip of adhesive tape. The tape was checked daily and repaired, if necessary. The animal was suspended by the swivel harness from a hook above the suspension cage, allowing free 360° rota-

movements by Opto-Varimex-Mini (Columbus Instruments).

Labelled amino acid infusion

L-[4.5−3H] leucine (170 Ci/mmol) was infused for 6h, 250μCi per 100g body mass for measurement of incorporation of radioactive label into myofibrillar and sarcoplasmic protein fraction.

Tissue preparation

24h after the last experimental procedure, the animals were anaesthetized by intraperitoneal injection of ketamin (Clysol, Gedeon Richter A.O. Budapest, Hungary) 2.5 mg/100g body mass and diazepam (Lab Renaudin, France) 2.5 mg/100g body mass and sacrificed. The gastrocnemius muscles was removed, trimmed clean of visible fat and connective tissue, weighed, frozen and stored in liquid nitrogen until further processing.

RNA isolation

For total RNA isolation, muscle samples were homogenized with an Ultra-Turrax homogenizer in Trizol (Life Technologies, Paisley, Scotland, UK). Other steps were performed as described in the manufacturer’s protocol (Life Technologies 1995). The purity and concentration of total RNA was assessed spectrophotometr-

ically. Northern blot analysis was used for testing the specificity of cDNA probes, whereas slot blot analysis was used for quantification of the specific RNA amount.

mRNA analyses

For Northern blotting, 30μg of total RNA was denatured in loading buffer, electrophoresed in a 1% agarose/formaldehyde gel, and transferred to a nylon membrane (GeneScreen Plus, Bio-
technology Systems, Boston, USA) with a standard procedure [4]. For slot blotting, 20μg of total RNA was spotted on a nylon mem-

brane using a vacuum filtration manifold (Minifold II, Schleicher and Schuell, Dassel, Germany) [22]. All the membranes were incubated in 0.05N NaOH for 5 min to bind the RNA to mem-

brane. Prehybridization was carried out in a solution containing 5 X SSC, 5 X Denhardt’s solution, 50% formamide, ssDNA 100pg/ ml, 50μM sodium phosphate pH 6.8, 10% dextran sulphate and 1% SDS for 2 h at 42°C. The RNA-cDNA hybridization was performed for 24h at 42°C using the solution containing the same components as the prehybridization solution and [32P] labelled cDNA probe labelled with a Ready-To-Go-DNA Labelling Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The collagen probes were α12, a 2.4 kb human cDNA for proα1(I)-chain mRNA, E6, a 2.4 kb human cDNA for proα1(III)-chain mRNA and HT21, a 2.6 kb human cDNA for the α1(IV). The rat lysyl oxidase (EC 1.4.3.13) probe was a 0.6 kb product of an EcoRI digest of the p13L-0 clone. 1668 bp long cDNA was used for rat MMP-2 RNA. 0.6 kb ECO RV insert in pbloopscript II plasmid of mouse was used for TIMP-1 and 1.7-kb ECO RI insert in pbloopscript II plasmid of mouse for TIMP-2. After the hybridization, the membranes were exposed to KodakX-Omat film (Eastman-Kodak, Rochester, NY, USA) at −70°C. Attained signals were analysed using densitom-

etry (Personel Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA). The signal obtained by hybridization with a 24 mer-

ligonucleotide for 18S ribosomal RNA was used to normalize RNA loading/transfer amount.

Separation of myofibrillar and sarcoplasmic protein

The muscle samples were homogenized in a buffer containing: 50 mM KCl; 10 mM K2HPO4; 1 mM EGTA; 1 mM MgCl2; 1 mM 2-dithiothreitol; at pH 7.0. The homogenates were centrifuged at 1000 g for 10 min, and the supernatant was taken as a sarcoplasmic fraction. The crude myofibrillar pellet was homogenized in the same buffer with 0.1 % Triton X-100 and centrifuged at 10000 g for 10 min. Myofibrillar fraction was extracted with 3 volumes 100 mM sodium pyrophosphate, 5 mM EGTA, 1 mM dithiothreitol (pH 8.5), and centrifuged after 30 min gentle shaking, and diluted with one volume glycerol and stored at -80 °C.

Fractional synthesis rate of muscle proteins

Synthesis of myofibrillar and sarcoplasmic protein was determined by Sugden and Fuller [33] and expressed as a percentage of protein synthesized per day. Fractional rate of protein synthesis (Ks) was calculated: Ks = 100x Sb/ Sa x t, where Sa is specific radioactivity of protein, and Sb is the protein bound leucine, t – incorporation time of radioactive label in days.

Protein assay

Muscle protein was assayed using the technique described by Bradford [2].

Statistics

Means and standard errors of means were calculated from individual values by standard procedures of Excel. The data were analysed by SAS, using the analysis of variance (ANOVA) and the Pearson correlation coefficients were calculated. Differences were considered significant at p < 0.05.

Results

During the first 3 weeks (0-3) gastrocnemius muscle weight of control group increased about 14 mg/day, and at the same time hindlimb suspension decreased the muscle growth by about 7 mg/day (Fig. 1). During 3 weeks of suspension motor activity of animals decreased significantly and stayed practically at the same level also after 1 week of reloading (Fig. 2). A positive correlation exists between gastrocnemius muscle mass and ambulatory activity of rats (r = 0.907). The motor activity remained at control level after 3 weeks of reloading (Fig. 2). There was also a positive correlation between gastrocnemius muscle mass and ambulatory activity of animals after 3 weeks of reloading (r = 0.872). Hindlimb grip strength decreased during suspension, stayed decreased also after 1 week of reloading and reached the control level after 3 weeks of reloading (Fig. 3). During hindlimb suspension the level of fibrillar type I and III collagen and network forming collagen type IV mRNA in gastrocnemius muscle decreased significantly in comparison with control group (Fig. 4). mRNA level of all 3 collagen types was lower in comparison with control group also 1 week after reloading (Fig. 4). As shown in Fig. 5, mRNA level of lysyl oxidase in gastrocnemius muscle did not change significantly during suspension and following reloading in comparison with control animals. mRNA level of matrix metalloproteinase-2 in gastrocnemius muscle increased significantly after 1 week of reloading.

It is well known that contractile apparatus of skeletal muscle and collagen network can impact muscle function. Connective tissue in skeletal muscle transfers the force from muscle fibres out to the fascia, to the tendon and bone. A period of time without weight bearing causes modifications of structure and the function mostly of ST fibres [11] in which an atrophy and a slow-to-fast transition are the most prominent [9, 35]. Unloading causes the cellular responses, like downregulation of anabolic signalling proteins and loading activates the anabolic effect [12, 28, 31, 30, 32]. It is still unclear how extracellular and myofibrillar components respond to the unloading and following reloading in FT skeletal muscles. It has been shown that muscle mass decreased dramatically during unloading, while tendon tissue mass was unchanged [14]. This difference in the response may show that tendon tissue is protected from rapid changes in tissue mass, but muscle is subjected to substantial and fast changes in tissue mass [14]. The present study shows that unloading caused atrophy of the gastrocnemius muscle is accompanied temporally by a decrease of the synthesis rate of muscle proteins as well as fibrillar type I, III, and network forming type IV collagen. Loading influenced ECM turnover rate, collagen synthesis and MMPs activity increased [18]. Adaptive capacity of tendon and muscle are regulated by altered gene expression and protein levels of fibrillar and network-forming collagens [21]. Recovery of motor activity and muscle strength after unloading also temporarily coincides with myofibrillar protein and synthesis rate of different types of collagen. Although unloading decreases selectively the mass of different types of skeletal muscles, particularly their myofibrillar protein content [8], and twitch characteristics, the present study showed that in FT muscles both fibrillar- and network forming collagens and myofibrillar protein synthesis decreased during unloading. Increase in synthesis of fibrillar as well as network forming collagens and myofibrillar proteins did not start after 1 week of reloading. After 3 weeks of reloading synthesis of collagen and myofibrillar proteins did not differ from the control group level. Loading contrary to the detrimental effects of unloading increased muscle.
mass, myofibrillar protein content, MyHC I isoforms relative content and maximum tetanic tension of rats [9]. Lysyl oxidase plays an important role in the formation and regeneration of ECM by oxidizing lysine residues in elastin and collagen, initiates the formation of covalent cross-linkages which stabilize fibrous proteins [16]. This ability of the FT muscle has a tendency to decrease during unloading as shown in the present study. MMPs provide degradation of ECM compounds, secreted or released in latent form and become activated in pericellular environments [34]. MMP-2 regulates the integrity and composition of the ECM in skeletal muscle, plays a role in myofibre proliferation and differentiation, the fibre healing after injury and is presented in

Fig. 6 Changes in mRNA level of matrix metalloproteinase-2 (a), tissue inhibitor of metalloproteinasises-1 (b), and -2 (c) in gastrocnemius muscle during suspension and following reloading in comparison with control group (100%). Values are mean ± standard error. 3 w susp – after 3 weeks of hindlimb suspension. 1 w, 3 w rel – after 1 week and 3 weeks of reloading. * − p<0.05 in comparison with control group. ** − p<0.01 in comparison with control group.

Fig. 7 Changes in total protein (a) and myofibrillar protein (b) fractional synthesis rate during unloading and following reloading. Values are mean ± standard error. 3 w susp – after 3 weeks of hindlimb suspension. 1 w, 3 w rel – after 1 week and 3 weeks of reloading. * − p<0.05 in comparison with control group. * * − p<0.01 in comparison with control group. * * * − p<0.001 in comparison with control group. # − p<0.05 in comparison with 3 weeks of suspension period. ### − p<0.001 in comparison with 3 weeks of suspension period.

Table 1 The effect of unloading and reloading on the myofibrillar protein content in gastrocnemius muscle (mg/muscle).

<table>
<thead>
<tr>
<th>Group</th>
<th>Before experiment (0)</th>
<th>After 3-weeks of hindlimb suspension</th>
<th>After 1-week reloading</th>
<th>After 3-weeks reloading</th>
</tr>
</thead>
<tbody>
<tr>
<td>contr</td>
<td>120 ± 2.4</td>
<td>159 ± 3.2</td>
<td>170 ± 3.5</td>
<td>190 ± 3.9</td>
</tr>
<tr>
<td>suspension</td>
<td>89 ± 1.9</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>reloading</td>
<td></td>
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</tbody>
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values are mean ± standard error.

* * * − p<0.001 in comparison with before experiment period.

### − p<0.001 in comparison with 3 weeks of hindlimb suspension period.
higher amounts in patients with inflammatory myopathies [3, 17]. As shown in our study a significant increase of the activity of MMP-1 after 3-weeks of suspension and MMP-2 occurs during the first week of reloading in FT muscle. TIMPs are proteins which inhibit ECM degradation [1, 19, 36]. In skeletal muscle all TIMPs can inhibit all MMPs, with the exception of TIMP-1, which is a poor inhibitor [1]. TIMP-2 mRNA level starts to increase after 1 week of reloading and a significant increase was obtained in FT muscles at the end of 3 weeks of reloading.

In addition, TIMPs can promote or inhibit cell growth, depending on the type of cell and the inducer [1, 36]. Above-mentioned quantitative and qualitative changes in the intramuscular connective tissue contribute to the deteriorated function and biomechanical properties of the unloaded skeletal muscle [15]. Above-mentioned standpoints are in good agreement with the results of the present study. The present study also shows that the decrease in synthesis rate of myofibrillar proteins, fibrillar and network forming collagen during unloading, and increase during reloading, are temporarily very similar in FT skeletal muscles. Decrease of gastrocnemius muscle mass and myofibrillar protein content during unloading and increase during reloading are in good accordance with the muscle strength and motor activity of animals. The present study shows that also in FT skeletal muscles, changes of ECM components and myofibrillar proteins are regulated in similar way during unloading and reloading. Accompanied changes in muscle strength and motor activity with unloading and reloading support the functional significance of these changes. In conclusion, unloading causes the decrease of specific mRNA levels of fibrillar type I and III, and network forming collagen type IV in fast-twitch skeletal muscles. Reloading enhanced the specific level of mRNA for fibrillar and network forming collagens. mRNA level for matrix metalloproteinase-2 increased during the first week of reloading and decreased in the third week of reloading. In the third week of reloading the mRNA level of tissue inhibitor of metalloproteinase-2 increased significantly. In the present study it is shown for the first time that changes in collagen expression in fast-twitch skeletal muscles are in good agreement with changes in myofibrillar protein synthesis rate during unloading and reloading. From the standpoint of sport practice and rehabilitation it is useful to know that alterations in extracellular matrix and myofibrillar apparatus in FT skeletal muscles are releated with changes in muscle strength and motor activity. From the viewpoint of exercise training the dynamics of state of ECM and contractile apparatus has an importance in determination of recovery period of skeletal muscle between loadings.

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### References


