THE EFFECT OF UNLOADING AND RELOADING ON THE EXTRACELLULAR MATRIX IN SKELETAL MUSCLE: CHANGES IN MUSCLE STRENGTH AND MOTOR ACTIVITY

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ABSTRACT: During three weeks of hindlimb suspension muscle mass decreased 36% ($p<0.05$) in Soleus (Sol) muscle, 17% ($p<0.05$) in Gastrocnemius (GM) and had tendences to decrease in plantaris (Pla) (15%) and in extensor digitorum longus (EDL) (8%) muscles. Hindlimb grip strength decreased gradually during three weeks of unloading. Specific mRNA level for type I collagen decreased during three weeks of unloading in Sol muscle by 28% ($p<0.05$) and in GM muscle by 34% ($p<0.05$). mRNA level for type III collagen decreased in Sol by 22% ($p<0.05$) and in GM by 51% ($p<0.001$). Non-fibrillar type IV collagen mRNA level decreased in both above-described muscles about 25% ($p<0.05$). Lysyl oxidase (LO) mRNA level decreased by 46% ($p<0.05$) during three weeks of unloading only in Sol muscle. Matrix metalloproteinase-2 (MMP-2) mRNA level increased during reloading period in Sol and GM muscles subsequently 28% ($p<0.05$) and 49% ($p<0.001$). During unloading the activity of tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) in slow-twitch (ST) and fast-twitch (FT) muscles changed in different directions: during first week of suspension, their expression decreased in Sol muscle by 31% ($p<0.05$) and increased in Pla and GM muscle subsequently by 24% ($p<0.05$) and 31% ($p<0.001$). The pretranslational level of changes in fibrillar and non-fibrillar collagen, MMP-2, LO, TIMP-1 and TIMP-2 -are shown for first time together with changes in muscle strength and motor activity during unloading and reloading

KEY WORDS: extracellular matrix, unloading, reloading, muscle strength, motor activity

INTRODUCTION

Disuse causes muscle atrophy by decreased protein synthesis and increased degradation. Reduction in muscle cell size is accompanied with the enhanced volume of connective tissue [27]. Impaired function of skeletal muscle occures not only due to atrophy, but also because of connective tissue, separating capillaries from fibers [11]. Although the amount of connective tissue increases during disuse, the gene expression of fibrillar collagens in slow-twitch (ST) muscle decreases in the very beginning of disuse [9]. Hindlimb unloading during 28 days did not cause significant changes in fibrillar collagen expression [21].

Lysyl oxidase and collagen types I, III and IV in skeletal muscle have been shown up-regulated rapidly at the pretranslational level after strenuous muscle activity [8], but the changes during unloading are still unclarified. Induction of the collagen chain synthesis and a lysyl oxidase (LO) initiated cross-linking of collagen in the extracellular matrix (ECM) is co-ordinated [4].

The effect of disuse on non-fibrillar type IV collagen is less studied. As type IV collagen represents only a small proportion of skeletal muscle collagens, measurement of total collagen synthesis does not reflect the changes in type IV collagen during unloading. It has been shown that unloading decreased collagen IV synthesis and degradation ratio in skeletal muscle but is strongly muscle specific [1].

Collagens' metabolism depends also from degradation prior to or after their secretion from the cell. Degradation may be proteolytic or phagocytotic. Matrix metalloproteinases (MMPs) are the main components degrading extracellular matrix (ECM) components. MMP-1 has the ability to cleave the native helical structure of collagens I and III [30] and MMP-2 degrades denatured collagen IV and digests fibrillar type I collagen [26,30]. Cytokines, growth factors and corticosteroids are known to induce or repress the transcription of MMP genes [13,20].

MMPs activity is inhibited by tissue inhibitors of metalloproteinases (TIMPs) which regulate MMP activity during tissue remodeling [7].

The purpose of this study was to assess the effect of three-week hindlimb suspension and following two-week reloading on the collagen I, III and IV expression, coordination of this process with lysyl oxidase, matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) on the pretranslational level. We also wanted to clarify relations between

abovementioned changes and changes in muscle mass, muscle strength and motor activity.

We hypothesized that three-week hindlimb suspension would decrease the expression of fibrillar and network-forming collagen and would increase their degradation. We also hypothesize that reloading helps to recover the collagen expression and decrease their degradation rate.

MATERIALS AND METHODS

Use of the animals was in accordance with European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes and was controlled by the Committee of Laboratory Animal Science of the University of Tartu.

The used animals were 16 weeks old (at the beginning of experiment) Wistar rats (National Laboratory Animal Centre, Kuopio, Finland). All the animals were housed in identical conditions in polycarbonated type III cages, at 21°C. They received diet [SDS-RM1(C)3/8, Witham, Essex, UK] and water ad libitum.

The rats were assigned to control, hindlimb-suspended for 1 and 3 weeks and hindlimb reloading group for 1 and 2 weeks. The size of each group was 10 animals. The reloading period consisted of free cage activity for two weeks after 3-week hindlimb suspension.

Hindlimb suspension procedure. A modification of the tail harness model of Morey-Holton and Globus [22] was used to suspend the hindlimbs of rats. The animals were anesthetized, such that the tail was easily manipulated. The skin of the tail was thoroughly cleaned with alcohol and dried. The proximal part of the tail was wrapped with breathing tape so that half of the tail remained uncovered, thereby allowing normal thermoregulatory processes to occur. The portion of exposed tail maintained normal color, indicating that blood flow was not compromised. A swivel harness was attached on 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° rotation. The height of the hook was adjusted so that only the front limbs were able to contact the cage. The size of cage was such that the animals could easily reach food and water without being able to touch the sides of cages with the hindlimbs. This form of suspension provides traction along the tail and does not cause any obvious lesions on the tail.

The measurement of strength and motor activity. The force and hindlimb grip strength was measured before and after hindlimb suspension and after recovery period with Grip Strength Meter 0167-004L (Columbus Instruments) and expressed as N per 100g of bwt. Motor activity was measured by the screening of the amounts of ambulatory and total (stereotypic) movements by Opto-Varimex-Mini (Columbus Instruments). Ambulatory activity characterizes movemental activity, total activity includes stereotypic (scratching, grooming, digging etc) non-ambulatory movements.

Tissue preparation. Twenty-four hours after the last experimental procedure, the animals were anesthetized by intraperitoneal injection of ketamin (Calysol, Gedeon Richter A.O., Budapest, Hungary) 2.5 mg/100 g bwt and diazepam (Lab Renaudin, France) 2.5 and sacrified. The gastrocnemius, plantaris, extensor digitorum longus and soleus muscles were removed, trimmed clean of visible fat and connective tissue, weighed, frozen and stored in liquid nitrogen until further processing. Due to the small size of soleus and extensor digitorum longus these muscles were pooled for mRNA analyses, so that one sample consisted of right and left muscle of the same animal.

RNA isolation. For total RNA isolation, muscle samples were homogenized with an Ultra-Turrax homogenizer in Trizol (Life Technologies, Painsley, 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 spectrophotometrically. 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, 30 μg of total RNA was denatured in loading buffer, electrophoresed in a 1 % agarose/formaldehyde gel, and transferred to a nylon membrane (GeneScreen Plus, Biotechnology Systems, Boston, USA) with a standard procedure [2]. For slot blotting, 20 μg of total RNA was spotted on a nylon membrane using a vacuum filtration manifold (Minifold II; Schleicher and Schuell, Dassel, Germany) [19]. All the membranes were incubated in 0.05 N NaOH for 5 min to bind the RNA to the membrane. Prehybridization was carried out in a solution

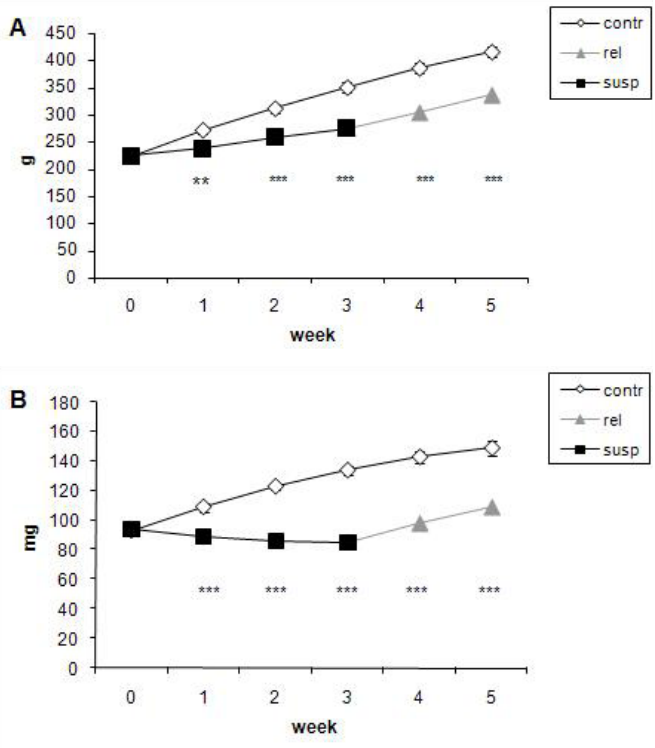

FIG. 1A-B. DYNAMICS OF BODY WEIGHT (A) AND MUSCLE SOLEUS MASS (B) DURING SUSPENSION AND RELOADING

Legend: 0 - before suspension, 3 - end of suspension, 5 - end of reloading, values are mean ± standard error
** - p<0.01; *** - p<0.001 in comparison with control group,
O - control group; rel - reloading; susp - suspension

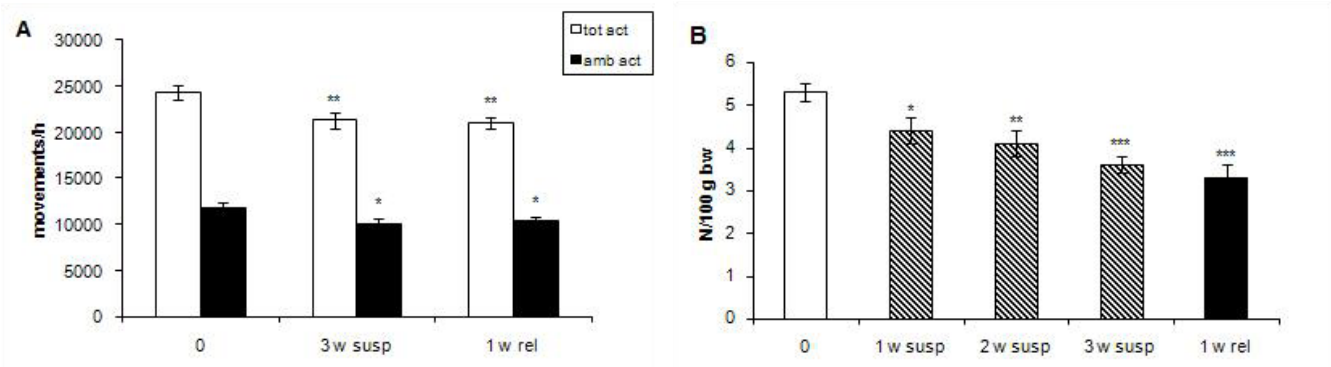

FIG. 2A-B. CHANGES IN MOTOR ACTIVITY (A) AND HINDLIMB GRIP STRENGTH (B) DURING 3 WEEKS IMMOBILIZATION AND FOLLOWING 1 WEEK RELOADING. AMBULATORY ACTIVITY CHARACTERIZES MOVEMENTAL ACTIVITY OF ANIMALS, TOTAL ACTIVITY INCLUDES STEREOTYPIC (SCRATCHING, GROOMING, DIGGING...) NON-AMBULATORY MOVEMENTS. GRIP STRENGTH OF HINDLIMB WAS USED FOR CHARACTERIZATION OF CHANGES IN MUSCLE STRENGTH DURING 3-WEEKS IMMOBILIZATION AND 1-WEEK RECOVERY AFTER IMMOBILIZATION.

Legend: values are mean ± standard error, * p< 0.05; ** p<0.01; *** p<0.001 in comparison with control group,
O - control group; rel - reloading; susp - suspension, tot act - total activity, amb act - ambulatory activity, 3w susp.- three weeks of hindlimb suspension, 1w rel.- one week of reloading after, hindlimb suspension

containing 5 X SSC, 5 X Denhardt's solution, 50% formamide, ssDNA 100 μg/ml, 50 mM sodium phosphate pH 6.8, 10% dextran sulphate and 1% SDS for 2 hrs at 42ºC. The RNA-cDNA hybridization was performed for 24 hrs at 42ºC using the solution containing the same components as the prehybridization solution and [32P] labeled cDNA probe labeled with a Ready-To-Go- DNA Labeling 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 pBluescript II plasmid of mouse was used for TIMP-1 and 1.7-kb ECO RI insert in pBluescript II plasmid of mouse for TIMP-2. After the hybridization, the membranes were exposed to KodakX-Omat film (Eastman-Kodak, Rochester, NY, USA) at -70ºC. Attained signals were analysed using densitometry (Personel Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA). The signal obtained by hybridization with a 24 mer oligonucleotide for 18S ribosomal RNA was used to normalize RNA loading/transfer amount.

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). Differences were considered significant at $p<0.05$.

RESULTS

During three weeks of hindlimb suspension the body mass did not increased as fast as in control group (Fig. 1A). Starting from the first week of suspension till the end of the recovery body mass was significantly lower than in control group. Muscle mass decreased during three weeks of suspension 36% ($p<0.05$) in Sol and 17 % ($p<0.05$) in GM and 15 % in Pla and 8% in EDL. Dynamics of soleus muscle growth is demonstrated in the Fig. 1B. Motor activity decreased significantly during 3 weeks of suspension and stayed on the same level even after one week of reloading (Fig. 2A). Muscle strength decreased gradually during three weeks of suspension and stayed on the same level in 1 week of reloading (Fig. 2B).

Specific mRNA level for type I collagen decreased during suspension period in Sol and GM muscles (Fig. 3A) and increased to the control group level during 2 weeks of recovery. mRNA level for type III collagen decreased during first week of suspension in Sol and Pla muscle and during one and three weeks of suspension in GM (Fig. 3B). During two weeks of reloading mRNA level for type III collagen increased in Pla and GM muscles in comparison with control group (Fig. 3B).

TABLE I. CHANGES IN MATRIX METALLOPROTEINASE 2, TISSUE INHIBITORS OF METALLOPROTEINASES 1 AND 2 mRNA IN SOLEUS (Sol), EXTENSOR DIGITORUM LONGUS (EDL), PLANTARIS (Pla) AND GASTROCNEMIUS (Gm) MUSCLE DURING HINDLIMB SUSPENSION AND FOLLOWING RELOADING

Group	n	muscle	MMP-2	TIMP-1	TIMP-2
hindlimb suspension 1 week	5	Sol	102 ± 9.4	69 ± 7.6*	88 ± 8.2
	5	EDL	97 ± 8.9	94 ± 8.7	103 ± 9.8
	10	Pla	98 ± 5.8	116 ± 10.2	104 ± 9.4
	10	Gm	108 ± 6.1	130 ± 11.0*	94 ± 5.7
hindlimb suspension 3 weeks	5	Sol	119 ± 10.6	120 ± 10.9	95 ± 9.7
	5	EDL	112 ± 11.1	101 ± 11.3	104 ± 11.2
	10	Pla	96 ± 6.8	124 ± 7.4*	95 ± 6.2
	10	Gm	114 ± 7.1	131 ± 8.1**	99 ± 6.7
1 week reloading after suspension	5	Sol	128 ± 10.8*	105 ± 10.3	107 ± 10.1
	5	EDL	99 ± 9.6	106 ± 9.5	98 ± 9.7
	10	Pla	115 ± 7.4	103 ± 6.9	104 ± 7.1
	10	Gm	125 ± 6.9*	108 ± 6.6	110 ± 6.8
2 weeks reloading after suspension	5	Sol	149 ± 16**	117 ± 10.6	104 ± 9.4
	5	EDL	116 ± 12	112 ± 10.4	101 ± 10.2
	10	Pla	92 ± 6.3	106 ± 6.9	92 ± 5.6
	10	Gm	110 ± 5.7	109 ± 6.1	119 ± 7.3*

Legend: Values are mean ± standard error; * - p<0.05, ** - p<0.01 in comparison with control group

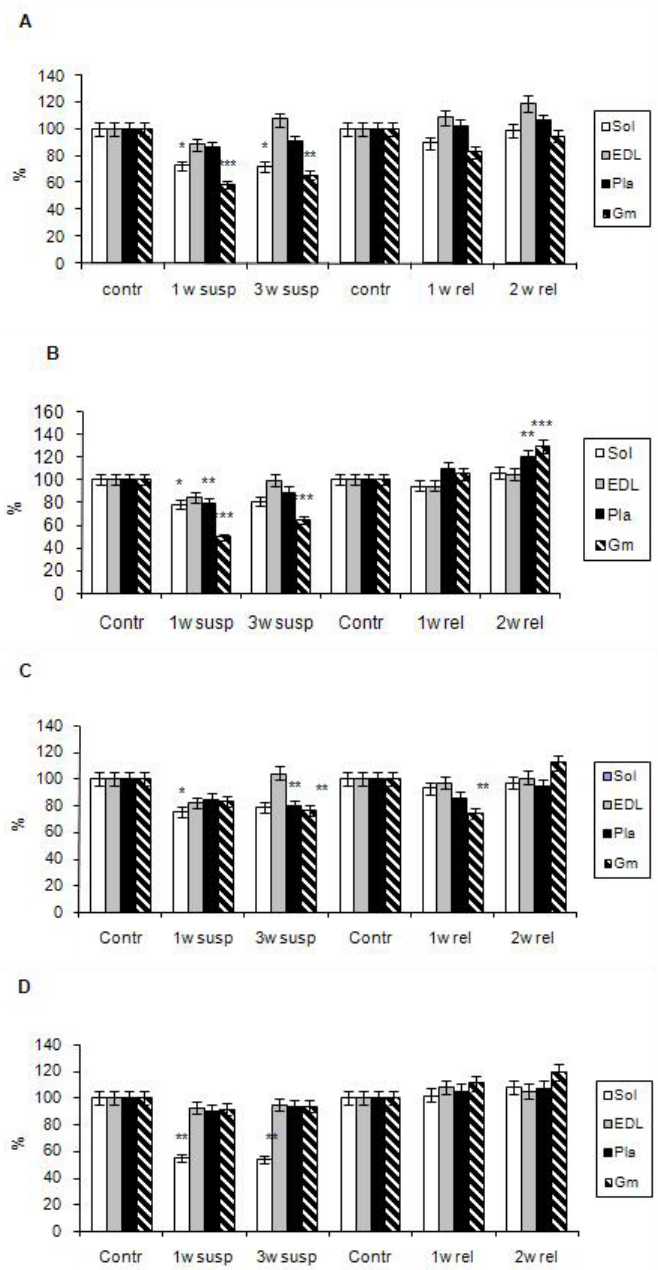

FIG. 3A-D. SPECIFIC MUSCULAR MRNA LEVELS FOR TYPE I (A), TYPE III (B), TYPE IV (C) AND LYSYL OXIDASE (D) IN SOLEUS (SOL), EXTENSOR DIGITORUM LONGUS (EDL), PLANTARIS (PLA) AND GASTROCNEMIUS (GM) DURING SUSPENSION FOR 1 AND 3 WEEKS AND FOLLOWING RELOADING FOR 1 AND 2 WEEKS.

Legend: values are mean ± standard error; n = 5 Sol, EDL; and n = 10 Pla, GM.
• - p<0.05; ** - p<0.01, *** - p<0.001 in comparison with control group
• - contr- control group
• - 1w susp - one week of hindlimb suspension
• - 3w susp - three weeks of hindlimb suspension
• - 1w rel – one week of reloading after hindlimb suspension
• - 2w rel – two weeks of reloading after hindlimb suspension

mRNA level for type IV collagen decreased during first week of suspension in Sol muscle and during third week of suspension in Pla and GM muscles (Fig. 3C). In GM muscle mRNA level for type IV collagen stayed on the low level during first week of reloading (Fig. 3C). mRNA level for lysyl oxidase (LO) decreased during three weeks of suspension only in Sol muscle (Fig. 3D) and increased during reloading period in comparison with control group in GM muscle (Fig. 3D). Matrix metalloproteinase 2 (MMP-2) mRNA level did not change significantly in skeletal muscles during reloading period in Sol and GM muscles (Table 1). mRNA level for tissue inhibitors of matrix metalloproteinases-1 (TIMP-1) decreased during first week of suspension in Sol muscle and increased in GM muscle during 1 and 3 weeks of suspension (Table 1). mRNA level of TIMP-2 increased in GM muscle after 2 weeks of reloading (Table 1).

DISCUSSION

Fibrillar type I and III collagens are most abundant in skeletal muscle epi- and perimyseum. Non-fibrillar type IV collagen is present only in basement membranes and has a critical role in the cellular arrangement of muscle tissue [17,18]. There are differences in collagen metabolism and content between muscles. Slow twitch (ST) muscles contain 40-50% more collagen than fast twitch (FT) muscles [16].

Reduced muscular activity decreases the collagen synthesis rate in skeletal muscle, immobilization downregulates the collagen synthesis at the pretranslational level, mainly among I and III collagens [9,29]. Unloading decreases selectively the mass of different muscles. One week spaceflight decreased Sol and GM muscle mass, but not the mass of EDL, and decrease of myofibrillar proteins in ST muscles was significantly greater than decrease of sarcoplasmic proteins [5]. Unloading has been shown to change muscle twitch characteristics. Decrease of myosin heavy chain (MyHC) I isoform expression and increase of MyHC IIb and IIx isoform expression in ST muscle improve abovementioned statement [31]. Unloading also induces a shift in the relative proportion of collagen isoform type I to III [21].

The present study shows that muscle mass of Sol and GM decreased significantly during hindlimb suspension and increased during two-week reloading. Muscle strength also decreased during hindlimb suspension, but this did not recovered during reloading period so fast as did muscle mass. Recovery of motor activity after hindlimb suspension is as fast as recovery of muscle strength. It is propably related with regeneration of muscle structures from disuse atrophy [10]. Decrease of collagen I mRNA level in Sol and GM muscle during three-week hindlimb suspension in present study shows that fibrillar collagen type I is more sensitive to unloading and the effect is much more longlasting than that of fibrillar type III collagen. This finding is in accordance with earlier findings where it was shown that hindlimb unloading induces reduction of collagen type I and is the reason of the slow-to-fast myofiber transformation in Sol muscle [21]. Reloading after hindlimb suspension in present study shows that collagen III mRNA level on the end of second week is higher than in control group. Two weeks of reloading have been shown to restore the Sol muscle metabolism [3]. Full recovery of ST muscle function via cross-sectional area and myonuclear domain size has been shown need more time for restoration of neural and mechanical properties of muscle [25].

Present study shows that non-fibrillar type IV collagen mRNA level is decreasing in both, ST and FT muscles during three weeks of

hindlimb suspension, but two weeks of reloading period is obviously not enough to restore the metabolic states of this collagen in muscle fiber basal lamina. It was previously shown that reorganization of basement membrane compounds needs certain time [15]. As type IV collagen plays a role in regenerative process on extracellular matrix (ECM), including matrix-associated signals and membrane-associated receptors that underline muscle fiber-matrix interactions, it shows how complicated is the evaluation of the functional significance of type IV collagen metabolism [28].

Lysyl oxidase (LO) which plays an important role in the formation and regeneration of ECM by oxidizing lysine residues in elastin and collagen, initiates the formation of covalent crosslinkages which stabilize fibrous proteins [12]. On this standpoint it is understandable that significant decrease in LO mRNA level was registered in this study only in Sol muscle.

Matrix metalloproteinases (MMPs) are providing degradation of ECM compounds [24]. MMP-2 mRNA level did not change significantly in the present study during three weeks of hindlimb suspension but increased in Sol muscle during two weeks reloading and in GM muscle after one week reloading. Tissue inhibitors of matrix metalloproteinases (TIMPs) are proteins which inhibit ECM degradation [6]. In the present study, the mRNA level of TIMP-1 decreased in Sol muscle after one-week hindlimb suspension, but increased in GM muscle during three-week suspension. TIMP-2 mRNA level increased in GM muscle, after two weeks reloading. As both intracellular (lysosomal phagocytosis) and extracellular degrading pathways (ECM proteinases) are present in degradation of skeletal muscle [14] during unloading and following reloading, it is complicated to put all the role to the MMPs in this process.

CONCLUSIONS

The changes of fibrillar and non-fibrillar collagen, LO, MMPs and TIMPs on the pretranslational level are shown for the first time together with changes in muscle strength and motor activity during three week of hindlimb suspension and following two week reloading. Biggest changes in specific mRNA level of type I, III and IV were registered in Sol and GM muscle, during three weeks of unloading. mRNA level of LO decreased also in Sol muscle. Significant increase in mRNA level for MMP-2 was registered in Sol muscle during reloading, showing that reaction of MMP-2 on the pretranslational level is not fast in all muscles. Changes in TIMP-1 mRNA level during first week of hindlimb suspension were contradictory in Sol and GM muscle.

The present study demonstrates that the metabolism of fibrillar and non-fibrillar collagens in ECM plays a crucial role in exercise and sport, influencing the strength development through transmission of contractile force in skeletal muscle.

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