J Musculoskelet Neuronal Interact 2006; 6(1):96-102

Original Article

Hylonome

Expression of matrix metalloproteinase 2 and heat shock protein-72 in immobilized muscle in rats

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Abstract

Metalloproteinases (MMPs) are proteolytic enzymes that function in the extracellular matrix to degrade connective tissues. While it is clear that certain induced skeletal muscle pathologies promote increased expression of MMP-2 and heat shock protein-72 (HSP-72), the relationship between muscle disuse and expression of MMP-2 and HSP-72 in muscles is unknown. These experiments tested the hypothesis that knee immobilization induced expression of MMP-2 and HSP-72 is disuse-dependent in a way that short-term joint immobilization increases HSP-72 expression, whereas long-term joint immobilization increases MMP-2 expression in skeletal muscles. Male rats (15-months-old) completed 1, 2, 3, and 4 weeks of knee joint immobilization. Muscle mRNA and protein levels of MMP-2 and HSP-72 were assessed in Gastrocnemius (Gast), Superficial and Deep Quadriceps, and Soleus (Sol) muscles by reverse transcriptase-polymerase chain reaction and western blotting, respectively. Results reveal that during the first two weeks of immobilization there is increased protein levels of HSP-72 and expression of mRNA of HSP-72 mainly in slow twitch muscle fibers. However, 3 and 4 weeks of joint immobilization increased both mRNA and protein levels of MMP-2 in skeletal muscles containing a high percentage of fast type II fibers (i.e., Gast and superficial quadriceps). These results support the hypothesis that different periods of muscle disuse induced different proteins expression, and that the influence of joint immobilization on the expression of HSP-72 in the short-term, and MMP-2 in the long ran is associated to fiber types.

Keywords: Immobilization, Atrophy, Matrix Metalloproteinase, Heat Shock Protein, Muscle

Introduction

Matrix metalloproteinases (MMPs) and heat shock proteins (HSPs) are highly conserved and constituent proteins in rodents as well as in human muscles, and characterized by an increase in their expression when myocells are exposed to a variety of stresses such as oxidation, denervation, overloading, or ischaemia[1,2]. However, their pattern of response to stress seems to be somewhat different. Whereas HSPs immediately and rapidly respond to stress such as increased temperature, the HSPs protect the inner cell[3] yet, in contrast, the MMPs will slowly and gradually respond to stress such as disuse muscle atrophy in order to protect the outer cell and the extra cellular matrix (ECM)[4]. Individual HSPs and MMPs have expression specific patterns in the individual tissues. The amount and the expression of HSP-72 and MMP-2 in skeletal muscle is fiber-type specific. HSP-72 appears to be proportional to the percentage of type I fibers in hind limb muscles such as soleus, tibialis anterior and deep quadriceps[5], whereas MMP-2 appears to be proportional to the percentage of type II fibers such as gastrocnemius and superficial quadriceps[6]. Thus, both proteins play an important role in the homeostasis in case of muscle fiber disruption.

Different models were done to study the expression of HSPs and MMPs in skeletal muscle under certain conditions and pathologies, and they seem to be closely activity-dependent of loading levels of the skeletal muscles[7-9].

The primary purpose of this study was to further examine

The authors have no conflict of interest.

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Accepted 10 May 2005

the effects of joint immobilization on HSP-72 and MMP-2 expression. We hypothesized that the HSP-72 and MMP-2 content levels in immobilized legs would be changed with the duration of immobilization time, and that the longer the disuse lasted there would be a larger response of these proteins.

Materials and methods

Animals. Male Wistar rats (15-month-old, n = 20) were used for the experiment. The animals were divided into 4 groups (n=5 each) which were immobilized for 1, 2, 3, and 4 weeks. In this study, there were no control animals, but in our previous publication the base levels of the same breed, non-treated animals were reported[6]. Rats were anesthetized by 60 mg/kg Ketamine HCl intra muscular, and 40 mg/kg Nembutal intra peritoneal (IP) injection. Antibiotic, 70 mg/kg Cefamizine injected with Nembutal. The external fixation model has been described by Bar-Shai et al.[6]. Briefly, rigid immobilization was achieved by inserting through the lateral plane of the femur and tibia two 0.8 mm diameter Kirschner wires which were then connected by two threaded brass rods to make a rigid frame. The rods were 4.8 mm in diameter and 33 mm in length and had a 13 mm slot cut longitudinally from both ends to contain the wires. The right knee joint was immobilized in 45° flexion. The overall weight of the device was 12 g. The contralateral intact hind limb muscles were used as internal controls.

Rats were sacrificed 1 week, 2 weeks, 3 weeks, and 30 days after the EF. Immediately after an IP injection of pentobarbitol sodium (200 mg/kg), the surgical procedure of carefully removing the right and left gastrocnemius (Gast), Soleus (Sol) and Superficial and Deep Quadriceps (SQuad/DQuad) was conducted. Muscles were weighed and frozen in isopentane chilled by liquid nitrogen (-192°C). The Animal Ethics Committees of Tel Aviv University approved this study with all experimental procedures used.

Tissue extract preparation. Muscles were homogenized (1:7, w/v) in extraction buffer 100mM Trisma-Hcl, pH 7.6, 200 mM Mg Cl2, 100 mM CaCl2 and 0.1% Triton X-100 at 4°C. After centrifugation (15,00 g), the supernatant was divided into aliquots, and protein concentration determined by Bradford assay against bovine albumin serum standard curve. Equal amounts of total protein loaded for Western blotting (100µg/lane), and mRNA for reverse transcription-polymerase chain reaction (RT-PCR) (50 µg/lane) were used.

RT – PCR. Total RNA was extracted from muscle samples as described previously[10]. RNA was isolated from 100mg muscle tissue using an EZ-RNA isolation kit (cat. 20-400-100, Biological Industries, Beit Haemek, Israel). The RNA was used as a template for RT-PCR reaction (Access Quick-™ RT-PCR system, Promega A1702) using MMP-2 primers: sense CCACATTCTGGCCTGAGCTCCC and anti-sense GATTTGATGCTTCCAAACTTCAC, and alpha-tubulin primers (as a reference): sense: ATCACAGGCAAGGAAGATGC and anti-sense: ATTGACATCTTTGGGGACCA (Sigma). HSP-72: sense TCGGGAACCATGAATAGAGG and anti-sense TTTGGAGAAAGGAGCAGCAT and alpha-tubulin primers as indicated above. The reaction products were run on 1.2% agarose gel.

Control blots were performed using only secondary antibody.

SDS-PAGE and Western blot analysis for MMP-2. One hundred mg muscle tissue was homogenized (20 sec homogenization and 10 sec pause x 3 times) in cold buffer containing 42mM Trizma base, 0.3M KCl, 2.5mM MgCl, 0.1% Triton x-100 and protease inhibitor cocktail (P-8340, Sigma), and centrifuged (10,000 x g for 10 minutes at 4°C). The supernatants were collected, and total protein concentration was measured using Bradford reagent (cat. 500-0006, Bio-Rad, Hercules, CA). Equal amounts of supernatants were suspended in protein sample buffer containing 5% beta-mercaptoethanol, vortexed, boiled and centrifuged. The supernatants were treated with 10% SDS-PAGE. Proteins from polyacrylamide gels were transferred onto nitrocellulose membranes. Blots were blocked with 2.5% skim milk (cat.170-6404, Bio-Rad) in PBST (PBS containing 0.05% Tween 20) for 1 hour, reacted with MMP-2 specific goat polyclonal antibody (sc-6838, Santa Cruz Biotechnology, CA) and alpha-tubulin specific mouse monoclonal IgG2a antibody (sc-5286, Santa Cruz Biotechnology, CA) for 1 hour, washed three times with 2.5% skim milk in PBST for 30 minutes (3 x 10 minutes), reacted with bovine anti-goat IgG-HRP (sc-2350, Santa Cruz Biotechnology, CA), or donkey anti-mouse IgG-HRP (sc-2314, Santa Cruz Biotechnology, CA) respectively, for 30 minutes, and washed once with 2.5% skim milk in PBST for 10 minutes and three times with PBST for 9 minutes (3 x 3 minutes). The membranes were developed using Super Signal West Pico chemiluminescent substrate (cat. 34080, Pierce Chemical Co., Santiago, Chile) followed by exposure to X-ray films (Fuji).

Measurement of HSP-72. To determine muscle levels of HSP-72, we performed polyacrylamide gel electrophoresis and immunoblotting using the techniques described by Powers et al. (1998)[7]. Briefly, muscle samples were homogenized and one-dimensional sodium dodecyl sulfate (12% SDS) polyacrylamide gel electrophoresis was performed to separate proteins by molecular weight. After separation, proteins transferred to nitrocellulose membranes (0.45 mm thick, BioRad, Hercules, California) using the Bio-Rad semi-dry transfer system at a constant voltage of 10 V for 20 minutes. After protein transfer, the nitrocellulose membranes was blocked for 2 hours using (0.5%) bovine serum albumin. Blots were incubated for 2 hours with alkaline phosphatase conjugated monoclonal antibodies specific for HSP-72 (SPA-810, SPA-815, Stress Gen, Victoria, Canada). The membranes then reacted with bromochloroindolyl phosphate-nitro blue tetrazolium substrate (Sigma Chemical, St. Louis).

Quantifications of the bands of HSP-72 and MMP-2 were performed using computerized densitometry of The Scion Image Version 4.0.2 beta, Scion Cooperation.

Immobilization period	1 week	2 weeks	3 weeks	4 weeks	% change	*p*	**exact p-value**
*Body weight (g)	275±13	273±15	270±15	269 ±14	-2	NS	**.167**
**Muscle weight (mg)							
Gast	1431±88	1400±72	1256±70	1132±91	- 21	0.05	**.833**
Sol	129±7	128±8	126±8	122±10	- 5.4	NS	**.387**
SQuad	673±41	631±40	558±36	510±32	- 24	0.05	**.667**
DQuad	795± 46	782±51	760±38	729±30	- 8.3	NS	**.255**

* Body weight is compared to an untreated control as previously measured[6].

** Compared with contra lateral leg. $p<0.05$; NS- Not significant. Values are mean values.

Table 1. Body and muscle weights (mean and SD).

Immobilization period	1 week		2 weeks		3 weeks		4 weeks		%	
Protein	MMP	HSP	MMP	HSP	MMP	HSP	MMP	HSP	**MMP	**HSP
Muscle										
Gast	47±4	75±7	55±5	84±8	119±10	60±5	*128±11	48±4	+57	-42
Sol	44±4	483±45	51±5	621±54	57±5	101±9	*61±6	33±3	+16	-95
SQuad	49±4	87±8	58±5	99±8	121±11	72±6	*143±13	32±2	+59	-67
DQuad	46±4	578±54	50±5	672±60	59±6	145±13	*68±6	44±3	+26	-94

Gast - Gastrocnemius; Sol- Soleus; SQuad – Superficial Quadriceps; DQuad –Deep Quadriceps

* between the fourth and second week of immobilization, a significant different from the contra lateral legs.

** comparison between week one and four. Data are the mean values (n= 5 in each group)

Table 2. Content of MMP-2 *(μg/mg muscle weight)* and HSP-72 *(expressed as a percentage of HSP-72 levels in contra lateral leg)* relative to muscle weight for the four different muscles following 1, 2, 3, and 4 weeks of immobilization. Alpha was set at $p<0.05$.

Statistical analysis

All statistical analyses were performed using a commercial software package (SPSS 10.0, SPSS Inc., Chicago, IL, USA). Chi square tests for cross-tabulation tables and Student's t-test were used to compare the immobilized leg to contra lateral legs in all the 4 groups. A one-way analysis of variance (ANOVA) and repeated measures that involved 2 independent variables (immobilization time, muscle fiber-type) were used to determine the effect of immobilization on the different muscles. Tukey's methods were used as a *post hoc* test. Statistical significance was accepted at an alpha level <0.05.

Results

Body and muscle weights. The effects of immobilization on body and muscle weights are reported in Table 1. Although no differences existed in animal body weights between the experimental groups at the beginning of the experiments, two, three and four weeks of immobilization resulted in a decrease in muscle weights, in particular, in predominantly type II muscle fibers (Gast weight reduced in 21%, $p<0.05$; SQuad weight reduced in 24%, $p<0.05$). Finally, records of both water and caloric intake of the animals during the experimental period revealed no group differences, suggesting that body or muscle weight differences were not due to dehydration or malnutrition.

To address the question of whether longer immobilization results in significant changes in muscle levels of MMP-2, and HSP-72, we measured muscle levels of both mRNA and protein for these important proteins.

MMP-2 mRNA levels increased in both the Gast (27.2%) (Figure 1) and Superficial Quad (30.2%) muscles, mainly following 3 and 4 weeks of immobilization. Consistent with the mRNA results, MMP-2 protein levels were also elevated in the Gast and Superficial Quad muscles following the immobilization period, and a total of 57% and 59% increased, $p<0.05$, respectively (Table 2). The relative content of MMP-2 in Sol and SQuad was increased 16% and 26%, p=NS, respectively.

The levels of HSP-72 for the four muscles are shown in Table 2. Hind limb immobilization for 1 and 2 weeks had a greater effect on mRNA of HSP-72 expression in Soleus (+91%, $p<0.05$) (Figure 2) and DQuad (+96%. $p<0.05$). The two other immobilization periods (i.e., for 3 and 4 weeks) resulted without a significant change in the HSP-72 content of

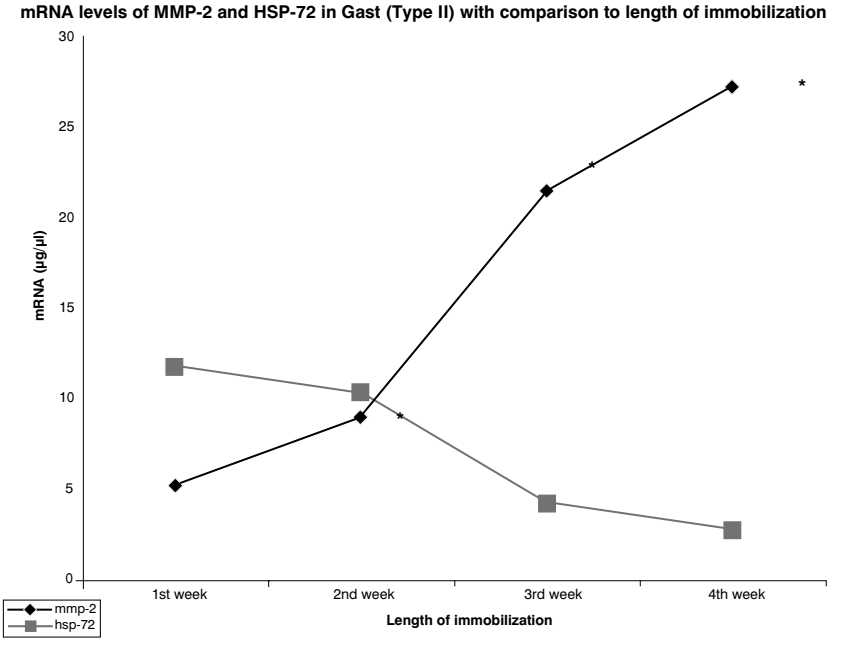

Figure 1. mRNA levels (µg/µl) of MMP-2 and HSP-72 in Gastrocnemius muscle (Type IIb) with comparison to length of immobilization (1, 2, 3 and 4 weeks).

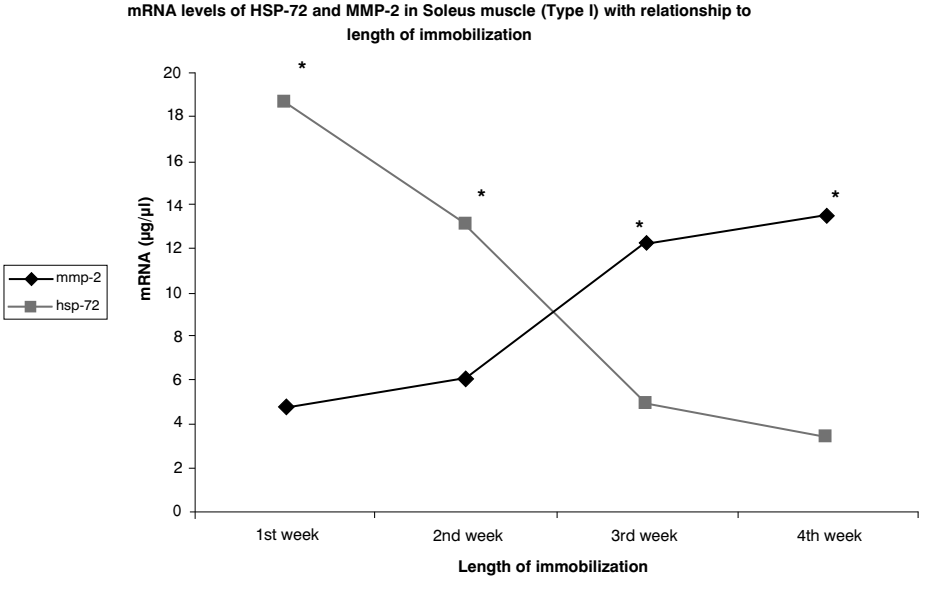

Figure 2. mRNA levels (µg/µl) of MMP-2 and HSP-72 in Soleus muscle (Type I) with comparison to length of immobilization (1, 2, 3 and 4 weeks).

all the muscles, compared with the contralateral leg.

Relationship between MMP-2 and HSP-72 content and muscle weight. Figures 3 and 4 show the relationship between the relative content of MMP-2 and HSP-72 and the four muscle weights following 1, 2, 3 and 4 weeks of immobilization. A negative linear correlation was observed between the levels of MMP-2 and the muscle weight, mainly for the two fast-twitch muscle fibers throughout the immobilization period (Gast, $r = -0.78$ $p<0.05$; DQuad. $r= -0.69$. $p< 0.05$), Figure 3.

Except for a weak correlation between Gast weight and HSP-72 ($r= +0.52$, $p =$NS), no correlation was observed between the levels of HSP-72 and the three muscle weights during the immobilization, yet a pattern of change in their correlation is clearly demonstrated in Figure 4.

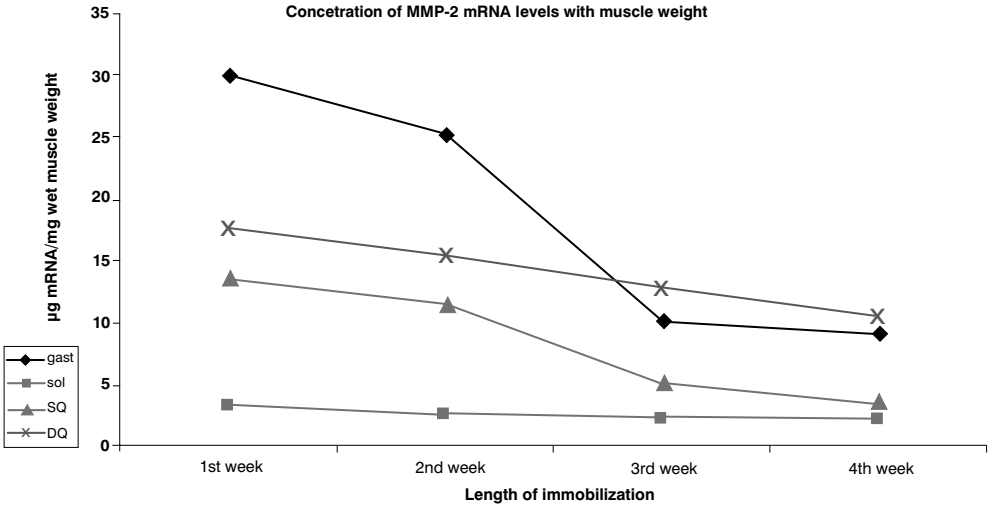

Figure 3. Concentration of MMP-2 mRNA levels with wet muscle weight (µg/mg) in Gastrocnemius (Gast), Soleus (Sol), Superficial Quadriceps (SQ) and Deep Quadriceps (DQ) with comparison to length of immobilization (1, 2, 3 and 4 weeks).

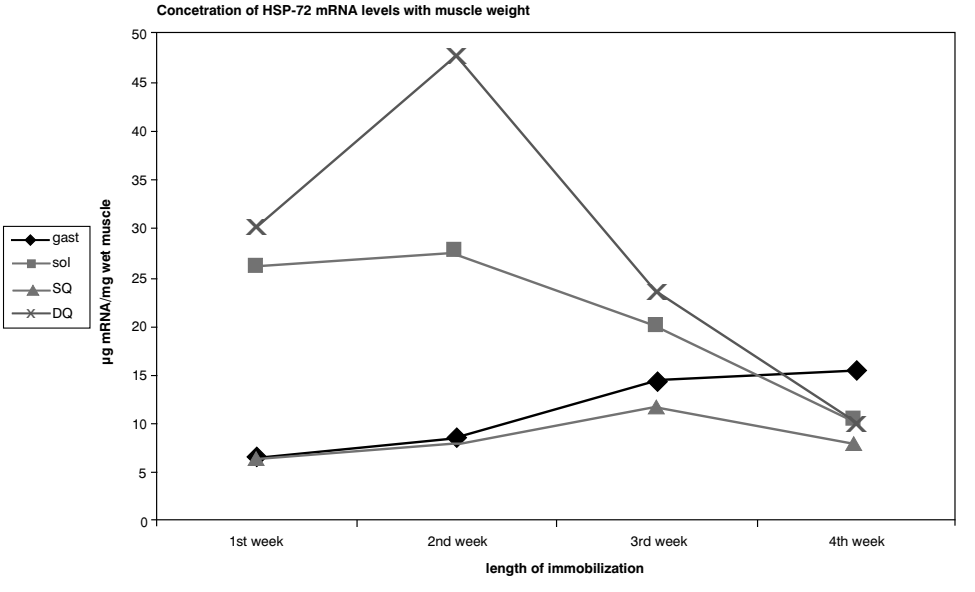

Figure 4. Concentration of HSP-72 mRNA levels with wet muscle weight (µg/mg) in Gastrocnemius (Gast), Soleus (Sol), Superficial Quadriceps (SQ) and Deep Quadriceps (DQ with comparison to length of immobilization (1, 2, 3 and 4 weeks).

Discussion

The general findings in this study were that the level of MMP-2 was higher in predominantly type II muscle fibers following 3 and 4 weeks of immobilization, whereas the level of HSP-72 was higher in predominantly type I muscle fibers following 1 and 2 weeks of immobilization.

It is worth noting that a knee joint fixation model does not promote a complete disuse model of the hind limb muscles. Knee immobilization primarily and directly affects the tight muscle, and indirectly affects the lower limb. Since weight bearing is still possible during the immobilization period there is a co-contraction of all the surrounding muscles, which elicited an electrical pulse via the motor nerve to the muscle fibers. Thus, our immobilization model, which only induces knee joint immobility, could not induce full muscle disuse.

The expression pattern of these proteins seems to be very different. Several reasons could be related to this different adaptation. First, the increase in MMP-2 content as the

immobilization lasted is related strongly to the amount of muscle mass atrophy. It appears that the significant changes in both Gast and SQuad weight (e.g., contain predominantly fast twitch fibers), and at the time, the increased of MMP-2 level, may explain that only after marked muscle mass atrophy, and presumably the least amount of knee joint movements, could support the view that the MMP-2 is muscle loading-dependent[11]. Type II, fast twitch glycolytic muscle fibers were the most affected by the immobilization as previously published[6]. Chronic disuse has been well-documented and resulting with decrease in the oxidative capacity (i.e., succinic dehydrogenase (SDH) and glutathione peroxidase (GPX) enzymes) of rat skeletal muscle. Second, we suggest that the immobilization protocol utilized in this study was probably sufficient to inhibit the glycolytic and the limited oxidative capacity exist in type II muscle that interferes with the myocell metabolism and therefore facilitates the protection activity of MMP-2.

In the present study, there was a significant change in the level of HSP-72 during the first two weeks of immobilization. Moreover, this adaptation happened only in type I muscle fibers (e.g., soleus and DQuad contain predominantly slow twitch fibers) despite the lack of change in the muscle weight. Since oxidative fibers of muscle contain the greatest concentration of mitochondria and thereby the greatest respiratory capacity, it provides the advantage of energy expenditure. However, during the condition of muscle disuse it would be expected in type I muscle to undergo 'use it or lose it' adaptation. Thus, the rapid response of HSP-72 to the outcomes of the disuse supports the notion that HSP-72 participates in the myocell homeostasis in case of muscle fiber disuse.

Our findings regarding HSP-72 are in contrast to the study published by Oishi et al.[12]. The disagreement might be explained by the 'tenotomy' model they used to induce inactivity. Such invasive procedure promoting 'inactivity' is more vigorous than the EF model we utilized, thus it might severely decrease neuromuscular activity through a reduction in muscle resting tone, functional decline of motor units and might cause impairments of local blood supply. The importance of the level of neuromuscular activity in determining the expression levels of HSP-72 has been demonstrated before[9]. The abrupt mechanical stress induced by 'tenotomy' does not allow muscle action potential and limb weight-bearing, thus leading to a more rapid response that accelerates the degree of muscle atrophy. In contrast, the EF model we used in this study gradually and progressively affects muscle fibers and therefore the adaptation mechanism is different. Progressive and rapid increase in HSP-72 protein was also observed in the rats' vastus lateralis muscle subjected to intensive exercise training[13] and continued electrical stimulation[14]. The amount of HSP-72 appears to be proportional to the percentage of type I fibers in hind limb muscles, with predominantly the soleus having the highest HSP-72 levels[5]. These data reflect that higher levels of HSP-72 are more sensitive to respond to different mechanical functions (i.e., disuse, overused).

In a more recent study Desplanches et al.[15], tested the hypothesis that mechanical unloading for 2 weeks would affect the constitutive HSP-72 expression in slow-twitch muscles to a greater extent than in fast-twitch muscles. It appeared that unloading resulted in a preferential atrophy of slow muscles such as soleus and adductor longus but with no change in HSP-72 level. Moreover, in this recent study, HSP-72 levels were significantly lower in fast muscles such as the extensor digitorum longus and plantaris, and did not change with mechanical unloading.

The observations relating to MMP-2 findings in atrophied fast-twitch, type II muscle fibers are in agreement with previous publications[16,17]. Under normal circumstances, expression of MMP-2 by skeletal muscle is largely restricted to small amounts, virtually non-detectable, and mainly confined to blood vessels and peripheral nerve fibers. In contrast, during pathological, inflammatory and regenerative processes, MMP-2 is up-regulated[18-20]. It was postulated that if species which present certain myopathy or inflammation process – as species subjected to immobilization (i.e., a non-specific pathology or inflammatory disease) – a similar alteration in MMP-2 expression might be found. Indeed, the results of this study confirmed the hypothesis that MMP-2 level is gradually up-regulated in response to experimentally long-term immobilized muscle and especially in type II muscle fibers. The significant positive correlation between muscle weight and MMP-2 levels is therefore an important finding especially in relation to sport activities such as sprinters and jumpers.

What is the role of a change in muscle inactivity and the response of HSP-72 and MMP-2 in resuming activity is yet to be investigated. This is an important issue because these molecules are load-stress inducible. Moreover, whether 'remobilization' of a muscle has a direct effect on these proteins content or activity is unknown.

In summary, this study presented a different pattern of protein adaptation to disuse. The expression of MMP-2 is demonstrated in type II muscle fibers particularly after 3 and 4 weeks of immobilization, and it is muscle weight-related, whereas the expression of HSP-72 is demonstrated in type I muscle fibers mainly after 1 and 2 weeks of immobilization, yet it is not muscle weight-related.

It is clear that the implementation of disuse, inactivity and deconditioning as a 'passive lifestyle' could result in a decline of muscle mass accompanied with a protein degradation that could lead to 'sarcopenia' and end up with functional debility.

Acknowledgements

We would like to express our thanks to the Anne and Eli Shapira Foundation, Portland, Oregon, USA, for their support this study.

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