Effects of Passive Stretching on Muscle Injury and HSP Expression during Recovery after Immobilization in Rats

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Key Words

Cast immobilization • Stretching exercise • Muscle damage • Heat shock protein

Abstract

Objective: Stretching exercise is known to induce muscle hypertrophy and is implicated in the modulation of muscle fiber behavior. We aim to determine whether stretching exercise is protective against reloading-induced muscle damage in immobilized rat soleus muscle. **Methods:** Rat hindlimbs in 54 eight-week-old male Wistar rats were immobilized by cast for 4 weeks, followed by reloading alone through normal ambulation in 24 (group NS) and after passive stretch in 25 rats (group S). Stretching exercise (30 min each day) lasted 6 days. To determine if passive stretching affects expression of heat shock proteins (HSPs) in rat soleus muscle during reloading following cast immobilization, the ratio of invading muscle fibers and HSPs expression were measured following cast removal. **Results:** The ratio of invading muscle fibers increased during the first and second days of reloading in group NS. Compared with reloading alone, stretching exercise reduced invading muscle fibers at most

time points following cast removal (group S). Additionally, expression of HSP25 and HSP72 increased with time during reloading only in the group without passive stretch (group NS). **Conclusion:** Following immobilization, in the rat soleus muscle passive stretching exercise protects against injury induced by reloading. Furthermore, the protection provided by passive stretch is independent of HSPs.

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Introduction

Immobilization is essential during the initial treatment of musculoskeletal injuries to allow for regeneration. Nonetheless, immobilization also results in rapid muscle atrophy, intramuscular fibrosis, loss of muscle extensibility and joint movement limitation [1–4]. Skeletal muscle disuse atrophy affects patients' quality of life, particularly in the absence of appropriate rehabilitation, and has a significant impact on health care costs.

Immobilization-induced muscle atrophy involves biochemical and morphofunctional alterations of type I and type II fibers [5], which indicate changes in fiber cross-

Fig. 1. Experimental design.

Fig. 2. Schema illustrating the method of stretching exercise. The stretching exercise was performed cyclically in the direction of plantar and dorsiflexion (in the range of 40° from the maximum of dorsiflexion) using a stretch apparatus. The stretching was applied once every 4 s. It was performed for 30 min each day, 6 days per week.

sectional area, fiber type composition and pathological fiber alteration [4, 6]. Independent of events leading to atrophy, reloading of the muscle is necessary to return to a pre-atrophy functional level.

Contrary to the volume of knowledge regarding immobilization, the effects of various forms of remobilization on immobilization-induced alterations are less well known, although the question is of utmost importance in rehabilitation medicine and sport medicine [4, 6, 7]. Stretching exercise is known to induce muscle hypertrophy and has been implicated in the modulation of muscle fiber behavior. In addition, stretching also helps to prevent muscle atrophy and maintains the range of motion in joints. Furthermore, we have found that short duration stretching for 30 min a day can facilitate recovery of both muscle strength and range of motion [8], suggesting that stretching exercise could be effective therapy for ameliorating disuse muscle atrophy. However, few clinical studies have focused on the effects of stretching exercise on repair of muscle damage caused by reloading.

We aim to determine if stretching exercise is protective against reloading-induced muscle damage in immobilized rat soleus muscle. In addition, since heat shock protein (HSP) has a cytoprotective effect on stressed muscle, and stretching exercise is known to result in HSP

induction in vitro [9], we hypothesize an increase in HSP expression within the atrophic rat muscles after stretching exercise.

Material and Methods

Animal Care and Treatment Protocol

Fifty-nine 8-week-old male Wistar rats were used in this study. The rats received standard laboratory chow and water ad libitum. Animals were housed in a temperature-controlled room ($23 \pm 2^\circ\text{C}$) with a 12 h light and 12 h dark cycle. The rats were randomly divided into a control group (group C, $n = 5$) and the experimental group (total $n = 54$; fig. 1). Rats in the experimental group were further divided into 3 groups. Animals assigned to the immobilization group received immobilization alone (group I, $n = 5$). Rats in the other 2 groups were immobilized for 4 weeks and then underwent 2 different remobilization programs. One group was allowed to move freely in their cages and underwent passive stretching exercise under anesthesia (group S, $n = 25$). The remaining group was allowed to move freely in their cages but stretching exercise was withheld (group NS, $n = 24$).

Animals in the experimental groups were anesthetized by intraperitoneal injection with pentobarbital sodium ($40 \text{ mg}\cdot\text{kg}^{-1}$ body mass) and bilateral ankles were fixed in full plantar flexion with a plaster cast for 4 weeks. The plaster cast, worn from the distal foot to above the knee joint, was changed as needed. Previous studies have confirmed that rat hindlimb cast immobilization causes skeletal muscle disuse atrophy and increased vulnerability

to muscle damage during reloading following limb disuse [8, 10, 11].

In order to evaluate the effects of stretching exercise, the animals in group S were anesthetized by intraperitoneal injection with pentobarbital sodium ($40 \text{ mg} \cdot \text{kg}^{-1}$ body mass) and soleus muscles of the rats were stretched using a custom-built stretch apparatus (fig. 2). The hindlimb was stabilized by securing the foot with tape to the platform which connected to a movable wire. The movable wire was connected to the shaft, the amplitude and frequency of stretch of which were controlled by the stepping motor (linear motor LU4B45SA-2; Oriental Motor Co. Ltd, Japan). The stretching exercise was performed once per 4 s using a range of 40° from maximum dorsiflexion, as measured with a goniometer. The cyclically stretching was applied for 30 min each day, 6 days per week. A previous report showed that daily sessions of passive stretching applied for 30 min to the soleus muscle of rats immobilized in the shortened position were enough to maintain the range of motion of the hindlimb joints [3]. Group S underwent the stretching exercise prior to reloading. The experimental procedures were approved by the ethics committee for animal experimentation at Nagoya University School of Health Science.

Histological Observation

The rats were sacrificed by intraperitoneal injection with an overdose of pentobarbital sodium on days 0, 1, 2, 3, 5 or 7 following cast removal (fig. 1) and the soleus muscles from both hindlimbs of each rat. One soleus muscle was frozen and stored immediately in isopentane cooled in dry ice for immunoblots. The contralateral muscle was cut in half and embedded. All samples were then frozen in isopentane cooled in dry ice and stored at -80°C . Serial cross sections ($7 \mu\text{m}$) were cut and stained with hematoxylin eosin (HE) to assess the muscle injury. The images were imported into a personal computer. They were evaluated using a light microscope. Five image files were selected utilizing a random number table. Injured muscle fiber was defined as an invading muscle fiber, displaying infiltration of over 2 nuclei, as described previously [12]. The number of invading muscle fibers and the total number of muscle fibers contained in the picture (picture area 1.8 mm^2 ; $1.5 \times 1.2 \text{ mm}$) were counted. The ratio of the number of invading muscle fibers to the total number of muscle fibers was employed on the index of muscle injury in this study.

Polyacrylamide Gel Electrophoresis and Immunoblotting

The remaining muscle samples were minced and homogenized in 0.01 M tris buffer (pH 7.6) containing EDTA. Total protein was determined via the Bradford technique, and protein concentration of the samples was adjusted to $1 \mu\text{g}/\mu\text{l}$. The resulting samples were subjected to Western blot analysis. Proteins were transferred and blocked. The blots were incubated overnight at 4°C with the appropriate primary antibodies: mouse monoclonal anti-Hsp72 (SPA-810; StressGen, Victoria, B.C., Canada) or mouse polyclonal anti-Hsp25 (SPA-801; StressGen). Horseradish peroxidase-conjugated anti-mouse IgGs (MBL, Nagoya, Japan) served as secondary antibodies. The membranes were developed with an enhanced chemiluminescence detection system (RPN2106; Amersham Biosciences, Piscataway, N.J., USA), which was used for visualization. Photographs were acquired with Light Capture (AE-6971; ATTO, Tokyo, Japan). Densitometry and quantification were performed employing a CS Analyzer (v 2.0; ATTO).

Statistics

The ratios of the number of invading muscle fibers to the total number of muscle fibers were analyzed with the χ^2 test. In terms of data obtained by biochemical analysis, the average value of group C was set to 1, subsequently the value of each group was calculated relative to 1. The differences between groups were expressed as multiples of increase in comparison with the values of group C. The significance of changes in the mean were tested with 1-way analysis of variance (ANOVA). Where appropriate, Scheffé's significant difference test was applied for post hoc comparisons. Statistical significance was established at $p < 0.05$.

Results

Effects of Stretching Exercise on the Rate of Invading Muscle Fibers/Total Muscle Fibers following Cast Removal

The soleus muscles of normal (non-immobilized) rats (group C) displayed no invading muscle fibers. In contrast, invading muscle fibers were evident in the immobilized soleus muscle (group I) at day 0 following cast removal (fig. 3, 4).

To assess the effect of stretching exercise on invading muscle fibers, the ratio of invading muscle fibers to total muscle fibers was examined in the NS and the S groups, respectively. As shown in figure 4, the ratio of invading muscle fibers in group NS was initially high at day 1 after cast removal. Subsequently, the number of invading muscle fibers gradually declined until day 5, but increased again at day 7. In contrast, the quantity of invading muscle fibers in group S exhibited marked reduction at day 1. Moreover, the ratio of invading muscle fibers in group S was significantly lower than that in group NS at days 2 and 7, respectively.

Time Course of HSP72 and HSP25 Protein Expression following Cast Removal

Figures 5 and 6 show summary data pertaining to the time course of HSP72 and HSP25 protein expression following cast removal. HSP72 expression in group I at day 0 following cast removal was scarcely detectable and this finding is consistent with that observed in group C (fig. 5a, b). HSP72 expression in group NS increased progressively with time and reached its highest level on day 7. In contrast, HSP72 expression in group S remained at a low level throughout the experimental period and no significant differences were detected. In a manner similar to that of HSP72 protein expression, HSP25 expression in group I was also scarcely detectable (fig. 6a, b). In group NS, HSP25 expression tended to increase at day 1,

Fig. 3. Transverse cryosections of the mid-belly region of rat soleus muscles. HE stain. **a** Control muscle (group C). **b** Immobilized muscle (group I). **c** Stretched muscle on day 1 following cast removal (group S). **d** Nonstretched muscle on day 1 following cast removal (group NS). Arrows indicate the invading muscle fibers. Scale bar = 50 μ m.

Fig. 4. Time course of change in the ratio of invading muscle fibers (number of invading muscle fibers/total muscle fibers) in soleus muscles following cast removal. Values are expressed as mean $\pm$ SEM. ^a $p < 0.05$, between groups C and I; ^b $p < 0.05$, between groups NS and S; ^c $p < 0.05$ vs. group I.

followed by a gradual increase until day 7. HSP25 expression in group S also increased progressively with time. However, the level of HSP25 expression in group S was low in comparison to that of group NS at all time points, with the exception of day 7. No significant differences were evident.

Discussion

To return a limb which has been immobilized back to a functional level, reloading of the skeletal muscle after immobilization is necessary. We observed an increase in the number of invading muscle fibers in the rat soleus muscles after immobilization (group NS), suggesting muscle damage occurred during reloading after immobi-

Fig. 5. Time course of HSP72 protein expression in soleus muscles following cast removal. **a** Portions of Western blots reacted with HSP72 antibody. **b** Graphical representation of HSP content obtained from densitometric scanning of the Western blots. Values are expressed as mean $\pm$ SEM. ^a $p < 0.05$ vs. control (group C); ^b $p < 0.05$ vs. group I.

Fig. 6. Time course of HSP25 protein expression in soleus muscles following cast removal. **a** Portions of Western blots reacted with HSP25 antibody. **b** Graphical representation of HSP content obtained from densitometric scanning of the Western blots. Values are expressed as mean $\pm$ SEM. ^a $p < 0.05$ vs. control (group C); ^b $p < 0.05$ vs. group I; ^c $p < 0.05$ between groups NS and S.

lization. To counteract this phenomenon, we performed intermittent stretching exercises prior to reloading and observed a reduced number of invading muscle fibers in the experimental group S, compared to the reloaded controls, group NS. This is the first investigation to show that

stretching exercise can attenuate muscle damage after 1 week of reloading.

Despite this novel finding, it is not the first study to employ stretching as a protective intervention in skeletal muscle. Previous studies have demonstrated that stretch-

ing exercises protect muscles from atrophy and muscle damage during disuse [13]. In addition, our finding of a decrease in muscle damage in the stretched reloaded group (group S) compared with the reloaded control group (group NS) is supported by Koh et al. [12] who found that a single bout of stretching protected skeletal muscle from contraction-induced injury.

In addition, we found that stretching exercises failed to elevate either HSP25 or HSP72 in reloaded soleus muscles (group S); however, both HSPs were elevated in the reloaded controls (group NS). This finding is contrary to our original hypothesis that an increase in HSP 25 and 72 would be seen in the atrophic rat muscles after stretching exercise prior to reloading. Our stretching exercise protocol, which is known to lessen reloading-induced injury, did not upregulate expression of either HSP when measured 24 h after in situ administration. This finding may indicate that induction of HSPs is not responsible for the observed protection, i.e. that HSPs may not play a pivotal role in protecting against muscle damage in our system. This conclusion, however, is contrary to evidence suggesting a role for HSPs in protecting muscle cells from stressors such as disuse atrophy [14], oxidative damage [15] and muscle damage [16–18]. Alternatively, we cannot exclude the possibility that an analysis performed 24 h after the stretching exercise is too long a period of time for a measurable induction of HSPs. Only 1 report in the literature states that HSP72 expression increased upon the application of cyclic stretching stimulation to skeletal muscle cells in vitro for 96 h [9]. This area of study is currently under investigation.

On the first day of reloading, induction of HSPs was more apparent in the NS group compared to the S group and, furthermore, expression of HSPs was progressively enhanced on several days following cast removal in the NS group. In a manner consistent with our results, Venojärvi et al. [19] demonstrated that HSP72 expression in immobilized rats was higher than in control (non-immobilized) rats during spontaneous recovery after immobilization. HSP72 is one of the most prominent members of the heat shock family of proteins, a family that also includes small HSPs such as HSP25 [20]. The mechanism by which increased levels of HSP72 provides cytoprotection in response to cellular stress appears linked to the direct regulation of specific cell signaling pathways by HSP72 [21–26]. One possible explanation, therefore, for the induction of HSPs in the NS group compared to the S group on the first day of reloading may be that it represents a reaction to the metabolic stress caused by the immobilization of the rat hindlimbs.

In conclusion, the present study shows that muscle injury response arises during reloading following long periods of immobilization and our passive stretching exercise protects against muscle injury. Furthermore, expression of both HSP25 and HSP72 increases during spontaneous recovery after immobilization, without the need for stretching exercise. These findings suggest that passive stretching is associated with the induction of adaptations that provide protection from reloading-induced muscle damage, independent of HSPs.

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