Molecular Biological Changes in Reloaded Skeletal Muscles after Rat Hindlimb Suspension

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Abstract. [Purpose] The purpose of this study was to clarify the expression changes of MyoD mRNA under reloading rat skeletal muscles after hindlimb suspension. [Subjects] The subjects were 27 Wistar male rats aged 8 weeks old. [Methods] They were randomly divided into a control group (n=5) and 4 experimental groups (n=5 to 6). The experimental groups were all submitted to 14 days of hindlimb suspension provoking muscle disuse atrophy, and afterwards they were assigned to no-reload hindlimb suspended group (HS) and 2, 7 and 14 days reloading groups (2D, 7D, 14D). [Results] The MyoD mRNA expression level increased in the HS group was significantly higher in the 2D reloading group and decreased to controls levels in the 7D reloading group. [Conclusion] The MyoD mRNA expression results suggest that reloading after hindlimb suspension stimulates satellite cell activation. Furthermore, the peak of MyoD mRNA expression was observed after 2 days of reloading.

Key words: Muscle disuse atrophy, Reloading, MyoD mRNA

INTRODUCTION

Muscle weakness of lower limbs resulting from disuse in prolonged rest, immobilization and hypogravity is very common in physiotherapy clinical practice. The patients with muscle disuse atrophy have impairments in their activities of daily living associated with increased fall risks. Therefore, prevention and treatment of muscle disuse atrophy are essential for quality of life1).

Researchers need to understand the physiopathology, the treatment and the prevention of disuse muscle atrophy. Gomes et al.2) and Tomori et al.3) showed that inhibition of muscle disuse atrophy is possible by passive extension of the muscle. In addition, Okita et al.4) and Yoshikawa et al.5) concluded that thermotherapy using hot (42 °C) and cold (10 °C) baths with rats as subjects had preventive effects on muscle disuse atrophy of the soleus. When Alley et al.6), Yamazaki7) and Itai et al.8) performed hindlimb reloading after a suspension period, they found effective recuperation from muscle atrophy. Yamazaki et al.9) consider weight-bearing stimulation less stressful and more beneficial than stretching and thermotherapy for patients.

Weight-bearing stimulus in hindlimb reloading stimulates the expression of growth factors, activating satellite cells. Once activated, satellite cells multiply by mitotic division and fuse to form new muscle fibers (muscle hyperplasia). The activated satellite cells can also fuse with damaged
fibers to repair them. Some satellite cells don’t fuse and remain latent till the next stimulation\(^{10}\).

When a satellite cell is activated, MyoD mRNA which is a muscle differentiation gene expressed. However, MyoD mRNA isn’t expressed when satellite cells are latent\(^{11}\). Therefore, MyoD mRNA, as an expression marker, can be used in quantitative evaluation of satellite cells activation.

Investigation of MyoD mRNA expression according to reloading time could contribute to the scientific evidence of hindlimb reloading efficacy in prevention and recuperation from disuse atrophy. The treatment in clinical practice could be improved in terms of intervention time and intensity, by clarifying the satellite cells’ reaction time and peak activity after reloading stimulation.

Although there have been many investigations into the effects of reloading after rat hindlimb suspension, they have mainly focused on histological changes such as muscle composition ratio and muscle wet weight. There are few studies that have investigated the increase and decrease of satellite cell activity, and there are fewer studies that have used MyoD mRNA as an expression marker to investigate the activity.

MyoD mRNA expression in muscle recovery after disuse atrophy has not been clarified. Therefore, the effects of muscle reloading on MyoD mRNA expression are also unclear.

In this study, we examined the reloading effects after hindlimb suspension from the viewpoints of histology and molecular biology of rat muscles.

**SUBJECTS AND METHODS**

All the experiments in this study were carried out with the approval of Kanazawa University Animal Experiment Committee.

The experimental animals were 27 male Wistar rats (average weight, 248.4 ± 9.2 g) of 8 weeks of age. We randomly divided them into 5 groups. The control group didn’t receive any specific procedure and was kept normally in cages for two weeks (CON; n=5). The experimental groups had their hindlimb suspended for two weeks, and they were then subdivided into no-reload, 2 days, 7 days, and 14 days of hindlimb reloading groups. The HS (n=6) group was hindlimb suspended, but not reloaded. The 2D (n=6) group was reloaded for two days after hindlimb suspension. The 7D (n=5) group was reloaded for seven days after hindlimb suspension. The 14D (n=5) group was reloaded for 14 days after hindlimb suspension.

The method used for hindlimb suspension was described by Yamazaki et al.\(^{9}\). There rats were made to wear jackets. The jacket had a steel bar inside along the back to restrain the rat’s trunk and tail movements. The jacket was connected to a wire tied above the cage creating a state of hindlimb suspension. The rats were able to move freely using their forelimbs in the cage. All animals were given food and water.

After hindlimb suspension, musculus soleus (SOL) and musculus extensor digitorum longus (EDL) presented disuse atrophy.

We performed anesthesia using pentobarbital sodium (50 mg/kg) injected to the abdominal cavity of each rat at the end of the suspension period and excised the bilateral SOL and EDL.

The muscles dissected from the right limb were weighed for wet weight on an electronic balance; then, they were rapidly frozen in isopentane liquid cooled to with liquid nitrogen. The samples were cryopreserved at −70 °C, and then sliced into 8 µm sections using a cryostat/microtome.

Hematoxylin and eosin staining was carried out on the muscle slides used for histological observation under an optical microscope. A digital camera for microscopes was used to take pictures of the slides. The images were exported to a personal computer and processed at 10 times magnification. Muscle fiber areas were measured using image analysis software (Scion Image). The cross-sectioned average area of 100 muscular fibers was measured for each muscle sample.

The left skeletal muscles were promptly dipped into RNA, later RNA Stabilization Reagent (QIAGEN), to synthesize cDNA (complementary DNA); then, they were incubated at 4 °C overnight to stabilize the RNA.

After removing the reagent solution, the skeletal muscles were stored at −70 °C until use. A portion of 20–30 mg of the muscle was cut from the middle of the muscle and was used to extract RNA which was performed using the RNeasy Mini Kit (QIAGEN) following the manufacturer’s protocol. In addition, DNAase I was used during extraction to raise the purity of RNA. The concentration and purity of RNA were tested by spectrophotometer with an absorbance ratio of 260/280 nm. The purification was acceptable when the absorbance 260/280 nm ratio was above 1.8. The RNA
concentration was obtained from the measurement results at 260 nm.

Fast strand cDNA was synthesized using the Prime Script 1st strand cDNA Synthesis Kit (TAKARA BIO) following the manufacturer’s recommendations.

The cDNA was 100 times diluted to perform RT-PCR (Reverse transcription polymerase chain reaction). Takara Ex Taq (TAKAR BIO) was used as a template for RT-PCR. The following primers were used: MyoD (5’-ACT ACA GCG GCG ACT CAGAC-3’ and 5’-ACT GTA GTA GGC GGC GT-3’) and were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The reaction condition per cycle in heat-annealing was: 95 °C for 2 minutes (initial denaturation), 95 °C for 20 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds, and the final extension reaction was at 72 °C for 5 min. The reaction was performed in 40 cycles for the MyoD measurement, and for GAPDH it was performed in 35 cycles.

The amplified product was analyzed by electrophoresis in 3% agarose gel using Mupid-2plus (TAKARA BIO) and stained using Gel Star (TAKARA BIO) for 30 min. After agarose gel staining, the images were viewed and photographed by an UV transilluminator.

The Real Time RT-PCR was performed using a Light Cycler system (ROCHE DIAGNOSTICS) and the SYBR Premix Ex Taq TM II reagents (TAKARA BIO). The primers were identical to those used in RT-PCR. The reaction condition per cycle of shuttle PCR was: 95 °C for 10 seconds (initial denaturation), 95 °C for 5 seconds (heat denaturation), and 60 °C for 20 seconds (annealing and extension). The temperature was increased for the melting temperature of the amplified product. The single peak in melting curve was confirmed. Each target mRNA was measured using a standard curve method. The GAPDH mRNA control value was subtracted from the MyoD mRNA values to normalize the values.

One way ANOVA was used to analyze the skeletal muscle wet weight, the muscle fiber cross-sectional area and the mRNA expression values. A Bonferroni multiple comparison was performed when the results of ANOVA detected significant difference. Multiple comparisons were examined in seven ways: CON vs. HS, 2D, 7D, 14D and HS vs. 2D, 7D, 14D. The level of significance was set at p<0.05. The data are displayed in terms of mean ± standard deviation.

**RESULTS**

Table 1 shows the muscle wet weights each group. The muscle wet weight of SOL in the HS group was significantly decreased in comparison to the CON group. The 2D group also showed lower values in comparison to CON; however the difference was not significant. Compared to the HS group, 7D and 14D had statistically higher average weights of SOL. The EDL average weight was significantly lower in CON than in HS. However there were no significant differences CON vs. 2D, CON vs. 7D and CON vs. 14D.

The mean cross-section area of SOL was significantly greater in CON than in HS and 2D. However the SOL mean cross-section area of CON was significantly lower than those of 7D and 14D.

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<tr>
<th>Table 1. Muscle Wet Weight (mg/g)</th>
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<td>CON (n=5)</td>
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<td>SOL</td>
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*: vs CON p<0.05, †: vs HS p<0.05.

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<th>Table 2. Muscle cross-sectional area (µm²)</th>
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<td>CON (n=5)</td>
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<td>SOL</td>
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*: vs CON p<0.05, †: vs HS p<0.05.
The mean cross-sectional area of EDL showed no statistical differences between the groups (Table 2).

Table 3 shows the expression levels of MyoD mRNA. The SOL MyoD mRNA expression in HS tended to increase compared to CON, but the difference was not significant. However in 2D, the expression was statistically higher than that of the CON group. 7D and 14D showed no significant differences compared with CON. The EDL MyoD mRNA expression of 7D was significantly decreased compared with CON, there was no significant difference between EDL MyoD mRNA expression of HS, and that of CON.

Figure 1 shows that RT-PCR bands were confirmed in all groups.

**DISCUSSION**

The Muscle disuse atrophy results from muscle fiber volume reduction due to the increase of proteolyses and from decrease of muscle fiber number after nuclear destruction. Muscle protein anabolism and catabolism homeostasis is affected in the disuse atrophy process. Decrease in protein synthesis is observed in rat skeletal muscles a few hours after hindlimb suspension, and in two weeks of hindlimb suspension, the catabolic rate of protein exceeds the anabolic rate. Therefore the main factor involved in muscle disuse atrophy is the protein catabolic progress.

In this study, a period of 2 weeks of hindlimb suspension was enforced. This hindlimb suspension time is reported to be sufficient to promote decrease in muscular fiber number and to activate muscle proteolyses.

Itai et al. measured muscle wet weight of 7-weeks-old rats after 16 days of hindlimb suspension. They also performed hindlimb reloading for 1 to 2 weeks and reported that SOL mass was restored. In this experiment, compared to CON, the SOL wet weight decreased in HS, had a similar value in 2D, and increased in 7D and 14D.

Mozdziak et al. performed 28 days of hindlimb suspension before 2 weeks of reloading with 48-day-old rats. After reloading, the diameter of SOL increased significantly in comparison to the suspension group. In our results, the SOL cross-sectional area compared to CON was significantly decreased in HS and 2D, but was increased in 7D and in 14D. These results suggest that recovery of skeletal muscle wet weight require a reloading period of longer than 7 days.

EDL atrophy was not found according to the wet weight and cross-sectional area after hindlimb suspension. Wineski et al. and Canu et al. reported that EDL is less influenced by hindlimb suspension compared to SOL.

In this study, HS muscle wet weight and skeletal muscle cross-section of SOL decreased significantly compared to CON, suggesting muscle atrophy of SOL. However, the muscle wet weight and skeletal muscle cross-section of EDL of HS was not decreased compared to CON. From these results, we speculate that muscle atrophy did not occur in EDL. From the histological investigations, reloading of more than 7 days was needed for improvement of disuse muscle atrophy of SOL.

Alway et al. reported increase in MyoD mRNA expression values of SOL after 21 days of hindlimb suspension in 4-month-old rats and after 14 days of hindlimb suspension in 39-month-old rats. In addition, Gallgly et al. showed MyoD mRNA
expression increase in 6-month-old rats after performing 14 days of hindlimb suspension. Reloading was not performed in these studies, but they showed that MyoD mRNA levels increases in unloaded conditions. In this study, the MyoD mRNA level in HS was higher than in CON, suggesting that disuse muscle atrophy by hindlimb suspension induced satellite cell activation.

According to Pierre et al., macrophages infiltrate skeletal muscle cells producing muscle fiber injury in hindlimb reloading after suspension. In contrast, Hawke et al. concluded that muscle fiber damage activates the expression of growth factors and stimulates fiber regeneration. Hindlimb suspension weakens the muscle fiber; therefore, subsegment reloading can easily induce muscle fiber injury.

Although the SOL weight and the cross section area were decreased in 2D compared to CON, the MyoD mRNA level in 2D was significantly increased compared to CON. The effects of hindlimb reloading on satellite cell activation were more evident on the second day. However, satellite cell activation did not occur concomitantly with histological improvement of the skeletal muscle.

MyoD mRNA is expressed with activation of satellite cells. It is known that MyoD plays a role in determining the fate of cells in myoblasts for muscle repair. McGeachie et al. and Roberts et al. propose that satellite cells are activated within 48 hours of skeletal muscle injury, and Robertson et al. consider that a myotube cell is formed 3–7 days after muscle injury. In this experiment, MyoD mRNA expression showed no further increases after 7 and 14 days of reloading. Furthermore, in the 7D and 14D groups we found no differences in muscle wet weight and cross-section area in comparison to the control, but increased values in comparison to the HS group. This suggests that on the seventh day of reloading, satellite cell activation is already finished.

In this study, we performed reloading after hindlimb suspension for disuse atrophy. MyoD mRNA showed a tendency to increase in the unloaded state. Additionally, MyoD mRNA increased significantly on the second day of reloading, but had returned to control levels on the seventh reloading day. The MyoD mRNA level did not change from the seventh to the fourteenth reloading day.

The results of this study suggest that satellite cells are activated by performing reloading after disuse muscle atrophy induced by hindlimb unloading, resulting in regeneration of skeletal muscle. For application to physical therapy clinical practice, we need to clarify the effective time of treat and rehabilitation for patients who develop disuse-related muscle weakness in the lower limbs.

REFERENCES