Effects of hindlimb immobilization on myonuclear number and DNA unit size

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Abstract

The decreases in myonuclei have been reported in several animal models including spaceflight, hindlimb unloading, spinal cord transaction, and denervation. However, little is yet known about the effects of hindlimb immobilization on the number of myonuclei. The present study was to investigate the adaptive response of myonuclear number and DNA unit size (cytoplasmic volume-to-myonucleus ratio) of soleus muscle fiber to the decreasing neuromuscular activity and chronic muscle shortening induced by hindlimb immobilization model. The DNA unit is a term describing the concept that each nucleus is a multinucleate muscle fiber which controls its surrounding cytoplasm. The right hindlimb was immobilized in a shortened position for 4 weeks and the left hindlimb served as control. Single muscle fibers were isolated from the soleus muscles by immersing them in alkali solution. After hindlimb immobilization, the body weight was slightly increased ($p<0.05$) whereas the soleus muscle wet weight was significantly decreased ($p<0.01$). The mean myonuclear number of the atrophied muscle remained unchanged compared with that of the control muscle, whereas the mean muscle fiber cross sectional area significantly ($p<0.01$) decreased. Therefore, the DNA unit size of the atrophied muscle was significantly ($p<0.01$) decreased compared with that of the control muscle. These findings suggest that after hindlimb immobilization, the DNA unit size decreases without a loss of myonuclei.

KEY WORDS : DNA unit; hindlimb immobilization; myonuclei

Introduction

Skeletal muscle fibers are the largest cells found in vertebrates and have hundreds or even thousands of myonuclei distributed along its entire length. The reasons for having so many nuclei within a single cell are not completely understood. The multinucleated nature of muscle fibers has lead to the concept that DNA unit[7], nuclear territory[11], nuclear domain[9] and myonuclear domain[2] describing the theoretical amount of cytoplasm are controlled by a single nucleus. The DNA unit size dynamically changes in response to physiological stimuli such as altered levels of muscle activation and mechanical loading. Muscle fibers have been shown to decrease in DNA unit
size concomitant with a loss of myonuclei under conditions of muscular atrophy induced by several disuse animal models including spaceflight[4, 10], hindlimb unloading[3, 12, 14], spinal cord transection[5, 8], and denervation[17, 22, 23].

Limb immobilization, another muscular atrophy model, is frequently used for treatment of musculoskeletal injuries and rapidly leads to physiological and biochemical alterations. Although this model (limb fixed in a shortened position) is also characterized by reduced neuromuscular activity and load bearing[20], it is apparently different from other disuse models in that muscle fibers can isometrically contract when fixed at a shortened length. Therefore, the adaptive responses of myonuclei and DNA unit size to limb immobilization may be different from other muscular atrophy models. Compared with the knowledge of other disuse models, little is yet known

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about the effects of hindlimb immobilization on myonuclear number and DNA unit size although this information is of utmost importance in sports and exercise physiology and contributes to rehabilitation medicine.

The purpose of the present study was to investigate adaptive response of myonuclear number and DNA unit size when the muscle of an adult mouse is fixed in a shortened position. The soleus muscle is chosen, because it is known to lose myonuclei resulting from several disuse models.

Materials and methods

Hindlimb immobilization

The experiments were conducted on 6-week-old male CD1 mice (n=6). The animals had free access to food and water and were kept in a thermostated environment (21℃) with constant day length (12 h). The right foot was immobilized in maximal plantar flexion, approximately 180 degrees between foot and leg, to fix the soleus muscle in a shortened position under ether anesthesia[19]. The left leg served as control. Great care was taken to make sure that the cast adequately immobilized the hindlimb without causing ischemia. The animals were sacrificed under ether anesthesia 1 month after hindlimb immobilization.

Preparation of single fiber

Single muscle fibers were isolated according to the methods developed by Wada and colleagues[23]. This technique is excellent becduse it is possible to obtain a large number of fine single muscle fibers. The hindlimb was excised under ether anesthesia, skinned, and immediately fixed in 4% paraformaldehyde solution containing 137 mM NaCl, 54 mM KCl, 5 mM $MgCl_2$・6 H_2O, 4 mM EGTA, and 5 mM HEPES, pH 7.4) for 7 days. After fixation, soleus muscle was carefully excised, trimmed clean of visible fat and connective tissues. Small muscle bundles were made by teasing whole muscle tissue with fine tweezers under a dissection microscope, which were trdhsferred and transferred to a microtube containing 10M NaOH solution for 3 hours at room temperature. The microtube was gently shaken for 10 minutes to separate the bundle into single muscle fibers. The single muscle fibers were immersed with phosphate-buffered saline (pH 7.2) and then mounted on gelatin-coated slides.

Nuclear staining

All procedures were carried out at room temperature. Before staining, the fiber was incubated in acetic buffer (pH 4.5) for 5 min. Myonuclei were stained with Mayer's hematoxylin, washed three times with acetic buffer, dehydrated through a graded ethanol series to absolute ethanol, and mounted with 100% glycerol.

Calculations

To obtain the nuclei counts, the total number of myonuclei in 250 μm were counted along the middle portion of each fiber segment using Axiophoto microscope (Carl Zeiss, Germany). The value could then be converted into myonuclei per mm because myonuclear density is relatively uniform along the length of the fibers[21]. The muscle fiber cross sectional area was calculated as $\pi \times$ (fiber segment diameter $/2)^2$ [14]. An estimate of the DNA unit size was made along the middle portion of each fiber segment after estimation of fiber segment volume from fiber segment diameter and length estimates [$\pi \times$ (fiber segment diameter$/2)^2 \times$(fiber segment length)/ myonuclei]. To correct for any differences in the state of stretch of the fibers, myonuclei per millimeter and CSA were corrected for differences in sarcomere length by multiplying the observed sarcomere length and dividing by 2.5 to normalize to a 2.5 μm sarcomere length. Muscle fibers with a sarcomere length of $>$ 3.3

μm or < 1.75 μm were omitted for analysis[16].

Statistical analysis

All data is expressed as means and SE. A student's *t*-test was used to evaluate the differences between control and hindlimb-immobilized animals. Pearson's productmoment correlation was calculated to evaluate the relationship between myonuclear number and cytoplasmic volume. Statistical significance was accepted at $p<0.05$.

Results

Body and muscle weights

A summary of the body weight and muscle weight is shown in Table 1. Body weight was slightly increased after 4 weeks of hindlimb immobilization. Muscle wet weight was decreased by 50% in the hindlimb-immobilized muscles compared with the control muscles.

Table 1. Body weight and soleus muscle wet weight

Body weight (g)		Muscle weight (mg)	
Before	After	Control	HI
38.7±0.9	39.4±1.1*	10.8±1.3	5.4±0.8**

Data are means ± SE. *Significant difference between before and after experiment ($p<0.05$)
**Significant difference between control and hindlimb-immobilized (HI) muscles ($p<0.01$)

Myonuclear number, fiber cross sectional area, and DNA unit size

Figure 1 shows single fibers from control and atrophied soleus muscles. A summary of the single fiber analysis is shown in Table 2. Mean myonuclear number of the atrophied muscle remains unaltered compared with that of the control muscle, whereas mean muscle fiber cross sectional area significantly ($p<0.01$) decreased. The DNA unit size of the atrophied muscle significantly ($p<0.01$) decreased compared with that of the control muscle. Figure 2

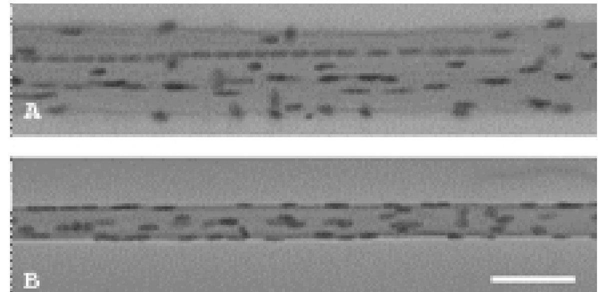

Figure 1. Hematoxylin-stained nuclei in mouse skeletal muscle following 4 week hindlimb immobilization. (A) Muscle fiber from the control muscle. (B) Muscle fiber from the hindlimb immobilized muscle. Calibration bar = 50 mm.

Table 2. Summary of single fiber analysis

	Control	HI
Fibers (n)	93	58
Myonuclei/mm	180.6±4.3	179.8±5.0
Fiber cross-sectional area (μm^2)	971.5±37.4	393.2±19.8**
DNA unit size (μm^3)	5504.3±204.4	2222.3±111.7**

Data are means ± SE. **Significant difference between control and hindlimb-immobilized (HI) muscles ($p<0.01$).

plots the cytoplasmic volume and myonuclear number for individual fibers from both control and atrophied muscles. The correlation between the cytoplasmic volume and myonuclear number was 0.395 ($p<0.001$) for the control and 0.341 ($p<0.01$) for the atrophied muscles.

Discussion

Differential experimental animal models for inducing muscular atrophy have been established. These models include spaceflight, hindlimb unloading, spinal cord isolation and denervation. These treatments induce a significant loss of myonuclei in response to decreased neuromuscular activity and/or mechanical loading. In this study, we found that the number of myonuclei of the hindlimb immobilized muscle remains unchanged, suggesting that adaptive response of myonuclei to hindlimb immobilization may be distinct from other models.

The decrease in body weight of the adult rat after hindlimb immobilization has been reported[1].

However, in our experimental model, the body weight was slightly increased and the animals are generally in good condition, suggesting that a stress reaction and/or decreased food intake is not a major contributory factor to the muscular atrophy. The reduction in soleus muscle mass after 4 weeks of hindlimb immobilization is consistent with the previous studies of rat soleus muscle fixed in plantar flexion[15, 19]. The reduction in fiber cross sectional area after 4 weeks of hindlimb immobilization in a shortened position is similar to the previous observation of rat soleus muscle fixed in a neutral position[1].

As shown in figure 2, it is evident that the distribution of myonuclear numbers of control muscle fibers is approximately similar to that of atrophied muscle fibers, suggesting that almost all muscle fibers maintain myonuclear number during hindlimb immobilization. This finding is inconsistent with previous studies using other disuse models. One possible reason could be the difference in satellite cell mitotic activity between other disuse models and hindlimb immobilization model. After hindlimb unloading by tail suspension, satellite cell mitotic activity is suppressed in the rat soleus muscle[13], indicating that there is no increment of nuclei derived from satellite cell proliferation in mature muscle fibers during hindlimb unloading. In contrast, the presence of myoblast and myotubes is observed during the first week of hindlimb immobilization[6], suggesting that satellite cells are activated, proliferate, and fuse into immature muscle fiber. If some nuclei derived from satellite cells were added to atrophied muscle fibers, myonuclear number could be constantly maintained during hindlimb immobilization. Further studies are needed to elucidate how muscle fiber maintains the number of myonuclei in the hindlimb immobilized muscle.

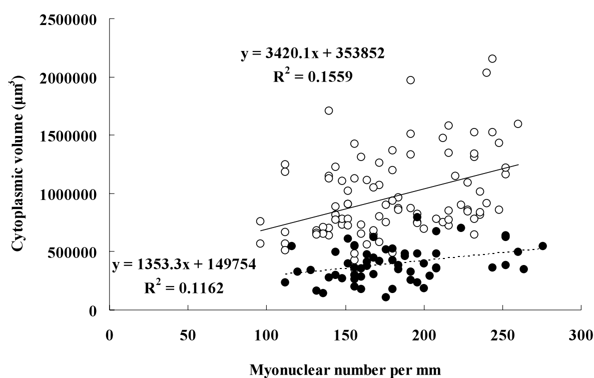

Figure 2. Relationship between cytoplasmic volume and myonuclei number per millimeter for control and hindlimb-immobilized muscles. Open symbols represent individual values from the control muscle fibers. Filled symbols represent individual values from the hindlimb-immobilized muscle fibers.

In this study, the DNA unit size decreased after 4 weeks of hindlimb immobilization in a shortened position. The DNA unit theory implies that each nucleus control a given volume of cytoplasm. A decrease in the DNA unit size may indicate that during adaptation to decreased neuromuscular activity and mechanical loading, either there is a cellular need for shorter diffusion distances for the mRNA or, alternatively, the nuclei may down-regulate transcriptional control and maintain a smaller cytoplasmic volume to myonucleus ratio.

The most important finding from this study is that DNA unit size decreased without a significant loss of myonuclei in response to decreased neuromuscular activity and chronic muscle shortening induced by hindlimb immobilization model. This finding indicates that the total pool of DNA available for transcription of muscle specific proteins is not affected and that myonuclear loss does not play a role in determing the muscle fibers size during hindlimb immobilization. However, short-term (6 days) limb immobilization also induces very little loss of myonuclei in rabbit skeletal muscle although muscle fibers maintain a constant DNA unit size[18]. In view of these findings, DNA unit size of muscle fiber could be modulated by two different steps. First, muscle fibers adapt to

external environments respond by eliminating myonuclei to maintain a constant DNA unit size during the early process of muscular atrophy. Second, during the later process of muscular atrophy, fiber size is consistently decreased to a great extent than myonuclear number, resulting in a significant decrease in DNA unit size.

Acknowledgements

This study was supported, in part, by the Grant-in-Aid for Scientific Research (B-17700499 to A. Wagatsuma) from Japan Society for the Promotion of Science, and the Grant-in-Aid for Scientific Research from the National Institute of Fitness and Sports of Japan (President's Discretionary Budget 2005, to A. Wagatsuma).

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ギプス固定がヒラメ筋の筋核数およびDNAユニットサイズに及ぼす影響

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要　　約

宇宙飛行や尾懸垂，脊髄切断，除神経等の動物モデルによって骨格筋は萎縮するとともに，筋核の減少が報告されている。しかしながら，ギプス固定による報告はほとんどない。そこで本研究では，ギプス固定がヒラメ筋の筋核数およびDNAユニットサイズに及ぼす影響を，マウス（CD1系雄性，６週齢）骨格筋を用いて検討した。DNAユニットサイズとは筋核が支配する細胞質容積のことであり，理論上考案された概念である。マウスの右足関節を最大限に底屈した状態で４週間ギプス固定した。左足は処置をせずコントロールとした。採取した筋は室温で４％パラホルムアルデヒドを用いて固定を行った。筋をリン酸緩衝液によって洗浄後，ピンセットにより小さな筋束にし，強アルカリ水溶液に室温でインキューベートした。筋束の入ったマイクロチューブをボルテックスにかけ，単一筋線維を採取した。筋線維をゼラチンコートしたスライドグラスに筋線維を並べ，ヘマトキシリン溶液を用いて核を染色し，顕微鏡下で観察，写真撮影を行った。ギプス固定後，体重は増加傾向を示したが（$p<0.05$），ヒラメ筋の湿重量は減少した（$p<0.01$）。また，ヒラメ筋の筋横断面積は減少したが（$p<0.01$），筋核数に変化は認められなかった。筋横断面積から推定された細胞質容積は筋萎縮により減少したため，DNAユニットサイズは減少した（$p<0.01$）。これらの結果は，ギプス固定により１つの筋核が支配する細胞質容積は減少するものの，同時に筋核の減少は伴わない可能性を示唆する。

KEY WORDS：DNA unit; hindlimb immobilization; myonuclei

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