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Influence of muscle length on muscle atrophy in the mouse tibialis anterior and soleus muscles

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ABSTRACT

The tibialis anterior and soleus muscles were fixed at the stretched or shortened positions to examine the influence of muscle length on muscle atrophy. Mice were divided into control (C), hindlimb suspension (HS), hindlimb suspension with ankle joint fixation at the maximum dorsiflexion (HSD), and hindlimb suspension with ankle joint fixation at the maximum plantarflexion (HSP). During the hindlimb suspension, the length of these muscles in the HS and HSP groups was very similar. Fourteen days after the hindlimb suspension, the atrophy of the tibialis anterior muscle in the HS and HSP groups was evidently milder than that in the HSD group, and that in the HS and HSP groups was very similar, suggesting that atrophy of the tibialis anterior muscle might largely depend on muscle length. Atrophy of the soleus muscle in the HSD group was milder than that in the HS and HSP groups, indicating that atrophy of the soleus muscle might also depend on muscle length. But atrophy of this muscle in the HSP group was milder than that in the HS group. These results demonstrate that some factors induced by the joint immobilization might be effective in preventing atrophy of the soleus muscle.

The length of muscles has been changed along with alterations in bone alignments after operations such as arthroplasty and limb lengthening and after joint immobilization by using a cast (6, 8, 12, 15). The changes of muscle length often induce the muscle atrophy, and some body functions might be disrupted (3, 9, 17). In the case of joint immobilization, when the ankle joint was fixed in dorsiflexion, the length of the tibialis anterior muscle was shortened and the soleus muscle was stretched. In contrast, if the ankle joint was fixed in plantarflexion, the tibialis anterior muscle was stretched and the soleus muscle was shortened. The muscle length in this case refers to whether the fixed muscle is stretched or shortened regardless of these contractions. Therefore, are there any differences in muscle atrophy in the fixed muscle at stretched and shortened positions? It was reported that muscle atrophy was milder, when the muscles were fixed in the stretched position, rather than in the shortened position (2, 7, 14). In these studies, the influence of muscle length on muscle atrophy was investigated in the stretched or shortened muscles, individually. However, the influence of joint immobilization on atrophy in the extensor and flexor muscles might be controversial when the joint was immobilized at any position. Therefore, in order to identify the influence of muscle length on muscle atrophy, extensor and flexor muscles should be examined concurrently.

In the present study, to stretch or shorten the leg muscles, the ankle joint was immobilized with a

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cast at the maximal dorsiflexion and plantarflexion positions during the hindlimb suspension, and the muscle wet weight and cross sectional area of muscle fibers in the tibialis anterior and soleus muscles were examined as parameters for muscle atrophy. The tibialis anterior muscle is considered to be fast muscle, and soleus muscle, to be slow muscle (19). There was speculation that the manner of muscle atrophy in the slow and fast muscles is different (4). In the present study, therefore, special attention was paid to the difference in the influence of muscle length on muscle atrophy between the fast and slow muscles.

MATERIALS AND METHODS

Materials. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine. Twenty male ddY mice (Japan SLC, Shizuoka, Japan) were used in the present study. The animals were randomly divided into four groups, with five mice per group: 1) control group (C), 2) hindlimb suspended without bilateral ankle joints immobilization (HS), 3) hindlimb suspended with bilateral ankle joints immobilization at maximum dorsiflexion (HSD), and 4) hindlimb suspended with bilateral ankle joints immobilization at maximum plantarflexion (HSP).

Hindlimb suspension. According to the method described by Morey (13), hindlimb suspension was applied to animals by suspending their tail for 14 days. Briefly, each animal belonging to the HS, HSD, and HSP groups was fitted with a tail harness and suspended by a string just high enough to prevent the hindlimbs from bearing weight on the floor or sides of the cage. The forelimbs were allowed to maintain contact with the floor of the cage and the animals were allowed to move freely. Therefore, the animals had full access to food and water. The animals in each group were housed in an isolated and environment controlled room at 25°C and 12:12-h light-dark cycle.

Ankle joint immobilization method. The animals in the HSD and HSP groups were lightly anesthetized by an intraperitoneal injection of pentobarbital sodium 50 mg/kg body weight (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) during the joint immobilization procedure. In the HSD group, bilateral ankle joints were immobilized at maximum dorsiflexion by using a plaster cast, thereby fixing the tibialis anterior muscle in a shortened position, and the soleus muscle in a stretched position. In the HSP group, the ankle joints were fixed at maximum plantarflexion, therefore, the tibialis anterior muscle was fixed in a stretched position, and the soleus muscle in a shortened position. The plaster casts were bandaged from the upper part of the knee joint to the forefoot to immobilize the ankle joints. The animal's toes were not wrapped with a cast, instead they were exposed to confirm the occurrence of edema. When loosening of the plaster cast or edema occurred, the plaster cast was immediately and properly rewound and replaced. The animals in the C and HS groups were both anesthetized at the same frequency as in the HSD and HSP groups to exclude the influence of anesthetic.

Morphological analyses. After the 14 days of hindlimb suspension, the animals were deeply anesthetized and subsequently their tibialis anterior and soleus muscles were surgically removed. Each muscle was cleaned away from the fat and connective tissues and their wet weight was measured. The animals were then euthanized by an intraperitoneal injection of a large dose of anesthesia. The muscle samples were embedded in optimum cutting temperature compound and tragacanth gum, immediately frozen in acetone cooled by dry ice, and stored at −80°C until used. From the middle part of the muscle belly of the tibialis anterior and soleus muscles, serial cross sections of 10 μm in thickness were cut with a cryostat (Shiraimatsu, Osaka, Japan) at −20°C, mounted on glass slides, and stained with hematoxylin and eosin for histological observation. For myofibrillar adenosine triphosphatase (ATPase) histochemistry, these sections were pretreated at pH 4.1 and pH 10.7 to categorize the muscle fiber as either type I or type II (10). Muscle fibers stained moderately with acid and alkaline ATPase preincubation were classified as type IIC (18). But type IIC fibers were hardly encountered in the tibialis anterior or soleus muscles. The sections for the ATP histochemistry pretreated at pH 4.1 were used to measure cross sectional areas of each muscle fiber type. Photographs (×200) were taken from each section with a digital camera (Olympus, Tokyo, Japan) attached to a light microscope (Olympus). At least 100 cross sectional areas of each muscle fiber type were measured, and statistically calculated by using the Scion Image (Scion Co., Maryland, USA).

Statistical analysis. Descriptive statistics included the mean and standard error of the mean values.

The measurements of the muscle wet weights and the cross sectional areas of the muscle fibers were analyzed by using a one-way analysis of variance. Significant differences between the four experimental groups were determined by Scheffe's post hoc test. The statistically significant level was set at $P < 0.05$.

RESULTS

Muscle wet weight

The mean values of the wet weight of the tibialis anterior muscle was 61.5 mg in the HS group, 59.7 mg in the HSP group, 50.9 mg in the HSD group, and 67.4 mg in the C group (Fig. 1A). The value in the HS and HSP groups appeared to be slightly less than that in the C group, but statistically insignificant. A statistical analysis revealed that this value of the tibialis anterior muscle in the HSD group was significantly less than that in the C group ($P < 0.001$). The value in the HS and HSP groups was almost the same. The mean values of the muscle wet weight of the soleus muscle was 9.5 mg in the HSD group, 6.8 mg in the HSP group, 4.8 mg in the HS group, and 9.7 mg in the C group (Fig. 1B). The value in the HSD group was almost the same as in the C group, and the value in the HS and HSP groups was significantly less than that in the C and HSD groups. Though statistically insignificant, the value in the HSP group appeared to be larger than that in the HS group.

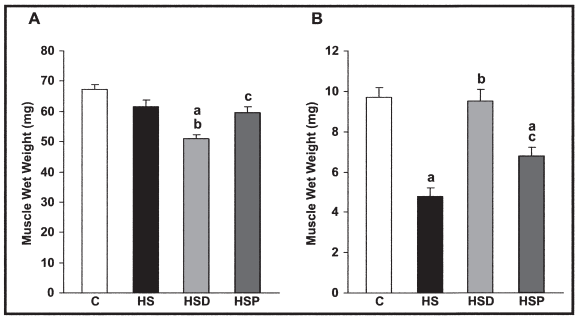

Fig. 1 Muscle wet weight (mg) of the tibialis anterior muscle (**A**) and soleus muscle (**B**). The wet weight of the tibialis anterior muscle was reduced most severely in the HSD group. In the soleus muscle, the wet weight was reduced in order of the HSD, HSP and HS groups. Values are means ± standard errors. [a] Significantly different from C, $P < 0.02$. [b] Significantly different from HS, $P < 0.01$. [c] Significantly different from HSD, $P < 0.01$. Abbreviations for Figs. 1–5: C, control group; HS, hindlimb suspended without bilateral ankle joints immobilization; HSD, hindlimb suspended with bilateral ankle joints immobilized at maximum dorsiflexion; HSP, hindlimb suspended with bilateral ankle joints immobilized at maximum plantarflexion.

Morphological appearance and cross sectional area of muscle fibers

In the cross section of the tibialis anterior muscle in the C group, muscle fibers were polygonal in shape, and inter-muscle fibers spaces were very narrow (Fig. 2A). Muscle fibers in the HS (Fig. 2B) and HSP (Fig. 2D) groups were slightly thinner, and in the HSD group (Fig. 2C), evidently thinner than those in the C group. The morphological appearance in the HS and HSP groups was very similar to each other. In the HSD group, the contour of muscle fibers became slightly rounded, and the inter-muscle fibers spaces widened. By ATPase histochemistry, when the sections were pretreated at pH 4.1, type I fibers were deeply stained, while type II fibers were lightly stained. In the tibialis anterior muscles, almost all fibers were type II, and there were hardly any type I fiber in any group (Figs. 2E–H). The muscle fiber cross sectional areas of the tibialis anterior muscle in the C, HSP, HS and HSD groups were 2474, 2181, 2131 and 1933 μm^2, respectively (Fig. 3A). The cross sectional area in the HS, HSD and HSP groups was significantly smaller than that in the C group ($P < 0.0001$), and this value in the HSD group was significantly smaller than that in the HS and HSP groups ($P < 0.02$). No significant difference in the value was noted between the HS and HSP groups.

Muscle fibers of the soleus muscle in the transverse sections stained with hematoxylin and eosin were polygonal in shape, and inter-muscle fibers spaces were hardly noticeable (Fig. 4A). The fibers in the HS (Fig. 4B), HSD (Fig. 4C) and HSP (Fig. 4D) groups were evidently thinner than those in the C group, and the inter-muscle fibers spaces were evident. The cross sectional areas of this muscle in the C, HSD, HSP and HS groups were 1809, 1652, 1457 and 1222 μm^2, respectively (Fig. 3B). The areas in the HS, HSD and HSP groups were significantly smaller than those in the C group ($P < 0.0001$), but the areas in the HSD and HSP groups were significantly larger than those in the HS group ($P < 0.0001$). By ATPase histochemistry, the soleus muscle was composed of type I fibers (47–56%) and type II fibers (34–44%) (Figs. 4E–H), and the ratio of these two types was almost the same in the four groups. Therefore, the cross sectional areas of the type I and type II fibers in the soleus muscle were individually compared among the four groups examined in the present study (Fig. 5). The mean values of the cross sectional ar-

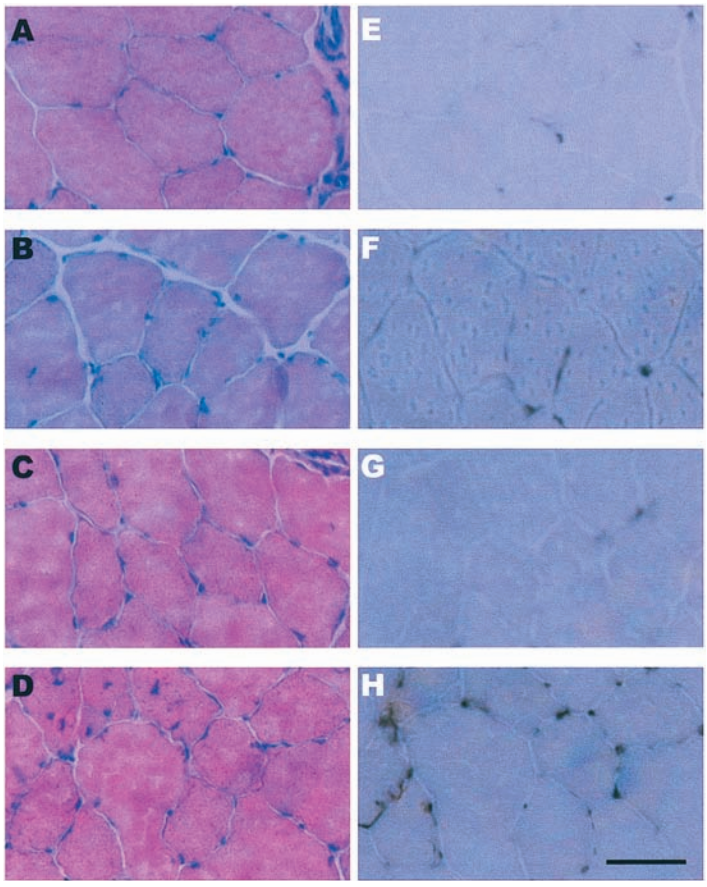

Fig. 2 Serial cross sections of the tibialis anterior muscle. Sections were assayed for hematoxylin and eosin (**A–D**) and myofibrillar adenosine triphosphatase (ATPase) histochemistry after being pretreated at pH 4.1 (**E–H**). With this ATPase method, type I fibers were deeply stained, while type II fibers were lightly stained. In this muscle, almost all fibers were lightly stained. The nuclei of muscular cells reacted positively and were located around the muscle membrane. A, E: C group; B, F: HS group; C, G: HSD group; D, H: HSP group. Bar = 50 μm.

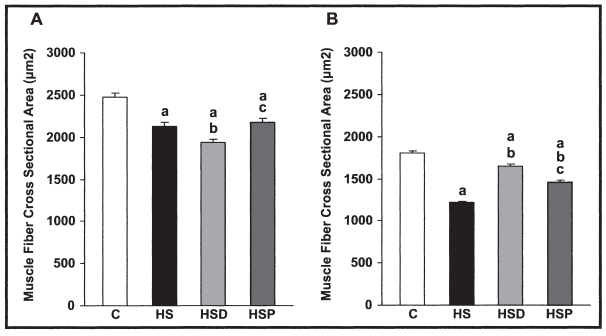

Fig. 3 Cross sectional area of muscle fibers of the tibialis anterior muscle (**A**) and soleus muscle (**B**). [a] Significantly different from C, $P < 0.001$. [b] Significantly different from HS, $P < 0.02$. [c] Significantly different from HSD, $P < 0.01$. In the tibialis anterior muscle, the value in the HSD group was significantly less than that in the HS and HSP groups, and that in the HS and HSP groups was very similar. In the soleus muscle, the area in the HS group was significantly less than that in the HSP group. Values are means ± standard errors.

eas of the type I and type II fibers in the HS, HSD and HSP groups were significantly smaller ($P < 0.05$) than those in the C group. Among the hindlimb suspension groups, these values of the type I and type II fibers in the HS group were significantly smaller than those in the HSD and HSP groups ($P < 0.0001$). There were no significant differences in the type I fibers between the HSD and HSP groups (Fig. 5A), while the cross sectional areas of the type II fibers in the HSD group were significantly larger ($P < 0.0001$) than those of the HSP group (Fig. 5B).

DISCUSSION

In the present study, the muscle wet weight and cross sectional area of muscle fibers were used as parameters to assess muscle atrophy. These two parameters of the tibialis anterior muscle in the HSD group were significantly less than those in the HSP

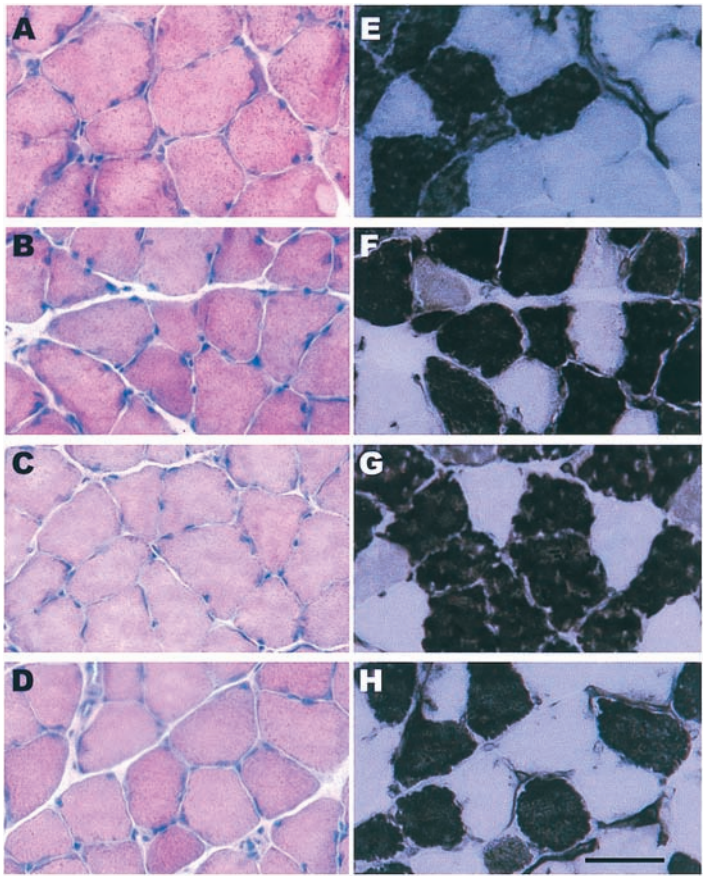

Fig. 4 Serial cross sections of the soleus muscle. Sections were assayed for hematoxylin and eosin (**A–D**) and myofibrillar adenosine triphosphatase (ATPase) histochemistry after being pretreated at pH 4.1 (**E–H**). With this ATPase method, type I fibers were deeply stained, while type II fibers were lightly stained. The nuclei of muscular cells reacted positively and were located around the muscle membrane. A, E: C group; B, F: HS group; C, G: HSD group; D, H: HSP group. Bar = 50 μm.

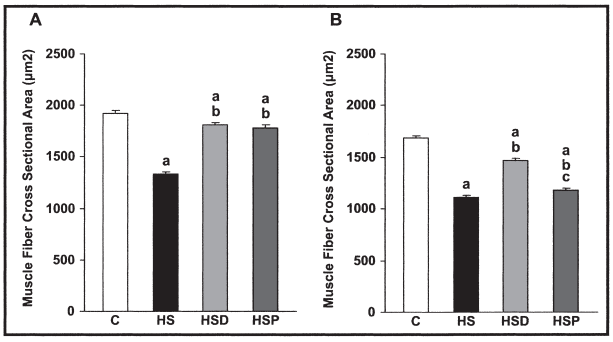

Fig. 5 Cross sectional area of type I fibers (**A**) and type II fibers (**B**) of the soleus muscle. [a] Significantly different from C, $P < 0.05$. [b] Significantly different from HS, $P < 0.05$. [c] Significantly different from HSD, $P < 0.0001$. The mean values of the cross sectional area of the type I fibers (**A**) in the HSD and HSP group were significantly larger than those in the HS group. On the contrary, the mean value of the cross sectional areas of the type II fibers (**B**) in the HSD group was significantly larger than that in the HS and HSP groups, and that in the HS and HSP groups was very similar. Values are means ± standard errors.

group. The tibialis anterior muscle in the HSP group was maximally stretched, and that in the HSD group was maximally shortened. When the hindlimb suspension was applied without any ankle joint immobilization, the ankle joint was kept in the plantarflexion position as described by other authors (11, 16). Accordingly, there might be only a few differences in length of the tibialis anterior muscle between the HS and HSP groups, and these parameters of this muscle in the HS and HSP groups were very similar. It has been said that the more a muscle was stretched, the more its atrophy was prevented (2, 7, 14). The finding suggests that muscle atrophy in the tibialis anterior muscle might largely depend on muscle length being kept during the hindlimb suspension. Muscle wet weight of the tibialis anterior muscle in the HSD group was significantly less than that in the C group. Though the cross sectional area of muscle fibers in the HS, HSD and HSP groups was smaller than that in the C group, the area in the

HS and HSP groups was larger than that in the HSD groups, suggesting that stretching might be effective to reduce muscle atrophy in the tibialis anterior muscle.

These two parameters of the soleus muscle in the HSD group were significantly larger than those in the HSP and HS groups, suggesting that muscle atrophy in this muscle might also depend on muscle length, as in the tibialis anterior muscle. But the wet weight in the HSP group was slightly larger, and the cross sectional area was significantly larger than that in the HS group. Since the muscle length in the HSP group was almost the same as in the HS group, or rather slightly shorter, the atrophy of the soleus muscle appeared to depend not only on muscle length but also on some other factors.

Witzmann *et al.* (20) reported that the force output of an isotonic contraction could be obtained at a velocity of 20–30% of the maximum velocity of fast fibers and at the maximum velocity of slow fibers. In the present study, ankle joint motion was hardly observed not only in the HSD and HSP groups, but also in the HS group during the hindlimb suspension, suggesting that the isotonic contraction might be too weak to prevent the atrophy of the tibialis anterior and soleus muscles. Baewer *et al.* (1) found that during the hindlimb suspension with the ankle joint immobilization, isometric contractile activity occurred in the soleus muscle, and suggested that muscle fiber atrophy was prevented by loaded muscle contraction. Fitts and Hurst (5) reported that isometric contraction resulted in greater protection from hindlimb suspension-induced atrophy of the slow muscle than that of the fast muscle. In the present study, ATPase histochemistry showed that almost all muscle fibers in the tibialis anterior were type II, and the soleus muscle contained type I (47–56%) and type II (34–44%) fibers. There were few differences in the ratio of these fiber types among the four groups in the present study. The cross sectional area of the type II fibers in the soleus muscle in the HSD group was significantly larger than that of the HS and HSP groups, and the area in the HS and HSP groups was almost the same. These findings suggest that the atrophy of the type II fibers in the soleus muscle depends largely on the muscle length being kept during the hindlimb suspension as the type II fibers in the tibialis anterior muscle. On the other hand, the cross sectional area of the type I fiber in the soleus muscle in the HS group was significantly smaller than that in the HSP group, and the area in the HSP group was almost the same as in the HSD group. These findings suggest that isometric contraction activities induced by the ankle joint immobilization might be effective in preventing the atrophy of type I fibers in the soleus muscle, and this might be one of the reasons why the muscle atrophy of the soleus muscle in the HSP group was milder than that in the HS group.

The findings of the present study suggest that although the fixed muscle length is an important factor in preventing muscle atrophy, joint immobilization with a cast which induces the isometric contraction might be also effective in preventing the atrophy of the slow muscle containing abundant type I fibers, such as the soleus muscle. Clinically, rehabilitation exercises should be planned in consideration of the muscle length, and should induce the isometric contraction exercise for the soleus muscle.

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